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KINETICS AND MECHANISM IN ACTIVATED SLUDGE PROCESSES

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PROGRESS REPORT
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Kinetics and Mechanism in Activated Sludge Processes

Period: September 1, 1962, to August 31, 1966

A. F. GAUDY, Jr.
Principal Investigator

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PROGRESS REPORT

Research Grant: Kinetics and Mechanism in Activated Sludge Processes, WP-00325
Principal Investigator: Anthony F. Gaudy, Jr., Ph.D., Professor, Director of Center for Water Research in Engineering
Sponsoring Institution: Oklahoma State University
Period Covered by Report: September 1, 1962--August 31, 1966
Date of Preparation of Report: August, 1966
Summary Statement:

The report consists mainly of a compilation of sixteen research papers and publications of work accomplished during the four-year period. Work on mechanism and kinetics of waste water purification was conducted in batch and in completely mixed continuous flow units. It was also necessary to conduct studies on various aspects of research methodology and technique. Reported in detail are investigations on the use of a colorimetric method for determination of COD (1, 1A), the use of COD values of bacterial sludges in waste water purification research (3) and a statistical study of the spot plate method for viable cell count (2). A considerable research effort was also made concerning the kinetics and mechanism of exertion of biochemical oxygen demand in both dilute and high energy systems (4, 5, 6, 7, 8, 9, 10, 11, 12). The effect of sludge concentration on various aspects of biological activity (13) and the mechanism and kinetics of substrate utilization at high biological solids concentration (15) were investigated. Studies were also conducted on the mechanism of substrate removal and regulation of nitrogen supply in treatment of nitrogen-deficient wastes (14).

Studies were also made to determine the applicability of various kinetic models and the degree to which steady state operation could be approached for heterogeneous populations (16).

Reference numbers refer to papers included in the report.

PROGRESS REPORT

KINETICS AND MECHANISM IN ACTIVATED SLUDGE PROCESSES

WP-00325

PREFACE

This report consists largely of a compilation of 16 copies of the original manuscripts of articles and papers which have been published or presented at research conferences concerning the work conducted on this research grant. The report covers the first four years of research findings on studies pertaining to the kinetics and mechanism of the activated sludge process. The report also covers, in summary fashion, work which is now nearing completion or is in progress. Additional reprints will be added to the report as they become available.

To date one Master's and two Doctoral theses have been completed by graduate students working on the project. The research on one Master's and two Doctoral theses is currently in progress.

The research grant has permitted investigations in considerable depth of the mechanisms and the kinetics of substrate removal and biological growth in discontinuous and in "steady state" continuous flow systems. In addition, a considerable amount of work has been accomplished on analytical methods and research methodology.

The research was initiated in August, 1962, with the aim of gaining a more basic understanding of the kinetics of substrate removal and the underlying physical, chemical, and biological mechanisms which govern substrate removal and concomitant waste water purification. The work is directly applicable to a more basic understanding of the activated sludge process. At the outset it was determined that a long-term, massive research effort was needed which would require multiple experimentation and the assessment of many system parameters and operational conditions. Work in discontinuous and in continuous systems has progressed concurrently, and has required various separate investigations into aspects of research methodology and analytical techniques.

The report which follows consists of a general discussion of work now in progress and work which has been completed during the past four years. The major portion of the report consists of a compilation of the original manuscripts of papers and publications of research supported wholly or in part by the grant. Work which has not yet been prepared for publication is discussed briefly, since a good deal of it awaits further analysis before conclusions can be drawn.

The major areas under investigation are discussed in summary form below. Two sections follow, the first containing an abstract of each paper and the second a copy of each paper. The latter are copies of the original manuscripts presented at meetings or submitted for publication. Except for minor editorial changes, they are identical to the published papers.

DETAILED SUMMARY OF WORK ACCOMPLISHED TO DATE

A. Long-term Kinetic Studies Conducted in Continuous Flow Units

Long-term kinetic studies were initiated at the outset of the project. These studies were conducted in a bank of laboratory activated sludge units. Preliminary experimentation was performed to determine the optimum design features of these units in order to obtain complete mixing with respect to substrate and biological solids. In these studies a soluble carbon source (glucose) was made the limiting nutrient, since the majority of wastes for which basic data are most critically needed are of industrial origin and consist in the main of soluble organic materials. Many of these wastes require the addition of nitrogen and phosphorus which are usually added in slight excess. Thus, for most cases of interest in the field, the carbon source is the limiting nutrient. Also, heterogeneous populations were used, since it is this type of population which is necessarily employed for waste water treatment. Because of the necessary utilization of heterogeneous populations (for which one can expect periodic fluctuation in predominating species even without exertion of any external factors), these studies have required a massive amount of experimentation. Indeed, the assessment of various changes in predominance which occur in heterogeneous populations has precipitated separate investigations in our laboratory (not directly associated with this research project) into possible ways and means of predicting species predominance. It was necessary in the study of mechanisms and kinetics applicable to heterogeneous populations, to obtain a great amount of data in order to perform adequate statistical analyses on the systems. Also, it was found necessary to perform multiple analyses in order to gain enough information to assess biochemical occurrences in light of the mechanisms which bring about or cause the various kinetic phenomena. It is well known that one of the objections some workers in the field have to the ultimate utility of some kinetic studies is their relative brevity and the fact that only one or two parameters are measured. It was intended in this study to overcome some of these pitfalls by obtaining enough experimental data to test various kinetic models or concepts which have been proposed and to seek more definitive expressions.

Many long-term experiments have been conducted with and without sludge recirculation, using a variety of dilution rates, or aeration times. Such parameters as biological solids concentration, effluent COD, effluent carbohydrate concentration, sludge protein and sludge carbohydrate have been determined. Physical operational conditions such as temperature and pH have been, in these studies, maintained as constant as possible, and in any event, have always been closely monitored. In all cases the continuous flow units were operated at each dilution rate for a sufficient length of time to provide reliable statistical estimates of the "steady state" parameters. For the series of long-term experiments employing no sludge recycle, various dilution rates, from 1/24 to the dilution rate at which cell dilute-out occurred were studied. Two separate series of experiments were run at inflowing substrate concentrations of 1000 and 3000 mg/l carbon source. In another series of experiments in which sludge recycle was practiced, dilution rates of 1/16, 1/4, 1/3, and 1/2 were used. A recirculation factor of 0.2 and a concentration factor of 1.5 were employed. Also, a long-term experiment was conducted in which total sludge recycle was practiced. In addition to the continuous flow experiments, cells were harvested from the units, and batch studies were made to determine relationships between logarithmic growth rate and substrate concentration for sludges developed at each dilution rate. This was necessary in order to provide an adequate test of the various formulations which have been suggested for relating sludge growth rate and substrate concentration.

Thus far, after nearly four years of experimentation and correlation of the results with the various hyperbolic function models, e.g., Teissier, Moser, Monod, as well as the two-phase linear concept employed by some workers in the pollution control field, it has been found that the most applicable expression correlating growth rate and substrate concentration for heterogeneous populations is the Monod relationship which was originally formulated using pure cultures. However, there are conditions under which other models could be employed to predict the levels of substrate and biological solids concentrations in the reactor without serious error. Also, it has been found that the maximum growth rate which is used as a constant in the Monod expression as well as in the other expressions, is not a constant for heterogeneous populations. In general, μ_m increases as the dilution rate is increased. As nearly as can be determined, this situation arises because of the selective pressure applied to the system due to the hydraulically-controlled growth rates which select against slower growing cells. Also, in the so-called steady state operation, considerable fluctuation in biological solids concentration was always found. Experimental evidence indicates that this fluctuation was not due to experimental error, but may be attributed to the fact that the yield "constant" is not a constant for heterogeneous populations growing on a single carbon source. This situation arises because of rather severe changes in predominance which occur during steady state operation at any given hydraulic or organic loading. It has also been found that, while the concentration of sludge may vary, other parameters, such as the level of COD and carbohydrate in the effluent, remain fairly constant. In comparing experimental data with the Monod kinetic model, it was found that for relatively low dilution rates the value for cell concentrations observed in the experiments fell fairly close to the values which could be theoretically calculated. However, as the dilution rate is increased, the theoretical curve and the experimental data diverge. It is important to note that these studies do not involve any aspects of shock loading or changes in the environment imposed on the system. These aspects are the subject of another research project (WP-00075).

A detailed report of the continuous flow studies for which carbon source was the limiting substrate is given in Paper 16 of the report, for work done at the 1000 mg/l inflowing substrate level. Presently, analysis and correlation of the results for the 3000 mg/l level and for studies in which cell recycle was used are in progress. This work requires further statistical analysis, and additional experimental runs of a spot-checking nature which will be completed during the coming year.

B. Studies on the Exertion of Biochemical Oxygen Demand

Originally it was intended to employ the BOD test as one of the parameters (along with the COD test and specific substrate analyses) to assess system efficiency during the conduct of both continuous flow and batch experimentation. However, at this time (1962) there was a considerable controversy in the field pertaining to a discontinuity in the kinetic course of BOD exertion, i.e., the generation of a plateau in the BOD curve and its significance in analyzing BOD data as well as its general significance in the course of waste water treatment in the activated sludge process. Some workers doubted that the plateau was of general occurrence, and there was no biologically sound explanation for the occurrence of the plateau. This militated against the utility of the BOD test as one of the parameters for use in research, and indicated that the test should be the subject of research rather than one of the means of accomplishing research. It was necessary to undertake investigations to determine whether the BOD test could be used in the present experimentation; and, since the exertion of biochemical oxygen demand is of extreme mechanistic and kinetic importance in the treatment of activated sludge, it was properly made one of the subjects of investigation in the current studies. In addition to the BOD test itself, some investigators in the field felt that occurrences in the BOD bottle were directly related to biological occurrences in the activated sludge tank where concentrations of cells and substrate are considerably higher. In the present studies investigations were undertaken in low energy systems (dilute systems - BOD bottle) and in high energy systems (at cell and substrate levels more commensurate with those used in the activated sludge process). The work is reported in detail in Papers 4 through 12. Through this work it was possible to show that the plateau in the exertion of BOD does exist over a wide range of seeding conditions and substrate concentrations as well as a wide range of types of substrate. Various theories explaining the occurrence of the plateau were formulated on the basis of the early studies, and were tested during the course of later investigations. This work also formed the basis of a decision not to employ the BOD test as one of the parameters routinely assessed in the present research. The work also led to definite experimental verification of the active role played by the protozoa in exerting the biochemical oxygen demand. Most of the existing theories had maintained that the protozoa played a passive rather than an active role in BOD exertion. Protozoa were thought to play a role in biochemical oxygen demand by bringing about a reduction in bacterial numbers; it was believed that this allowed a re-growth of bacteria and a concomitant increase in oxygen utilization. In the course of the present studies it was possible to show that the protozoa contribute directly to oxygen uptake, and that the increased oxygen uptake was due to the respiratory activities of the protozoa during the period when they were actively metabolizing the bacteria as a carbon source. Since similar results were obtained in both the BOD bottle and in systems with solids and substrate levels more comparable to those in the activated sludge process, it can be concluded that a portion of the oxygen requirement for activated sludge may be attributable to the metabolism of predators.

C. Role of Solids Concentration in Determining the Kinetics and Mechanisms of Substrate Removal

Investigations were conducted concerning the effect of sludge concentration on various aspects of biological activity in activated sludge; these included the relationship between rate of substrate removal and initial biological solids concentration, cell yield, and the percentage of theoretical oxygen demand exerted at the time of substrate and COD removal. It was found (see Paper 13) that the relationship between the rate of substrate removal and initial biological solids concentration was linear for a single sludge, but the value of the slope of the relationship depended not solely upon the type of substrate, but upon predominating microbial species. The sludge yield varied between 48 and 82% for single substrate, glucose. Concerning the percentage of theoretical oxygen demand exerted at the time of glucose removal, the results were in general accord with other results reported in the literature with respect to COD but not with respect to substrate removal as measured by a specific test for carbohydrate. A theory and experimental evidence of its validity were presented to explain the results.

Since the highest biological solids concentration employed in the first series of studies was approximately 2000 mg/l, it was felt that further studies at higher solids concentrations were also warranted. In addition, there was still much controversy over the mechanism of purification during rapid substrate removal, i.e., adsorption of soluble substrate on the floc, as opposed to metabolism, as the primary mechanism of rapid substrate removal. Therefore, studies were conducted at solids concentrations above 2000 mg/l in which cell yield, substrate removal rates, and initial uptake of substrate were determined (see Paper 15). The results obtained in this study offered no evidence for adsorption as a primary mechanism of substrate removal, but did offer additional evidence for the purely biochemical mechanism. The results offer further proof of the hypothesis presented by Rao and Gaudy (see Paper 13) for the different mechanisms of substrate removal for low and high solids systems. The results offer in addition a biochemical explanation for the occurrence of linear rates of sludge accumulation and substrate removal, and for the change in the percent of the theoretical oxygen demand exerted at the time of substrate removal at higher solids concentrations.

D. Studies on Substrate Removal and Regulation of Nitrogen Supply for Nitrogen-Deficient Wastes

Studies which were conducted on effects of solids concentration on kinetics and mechanism of substrate removal indicated that at high solids concentrations all of the substrate could be removed before the cells produced new protein. These studies, in turn, indicated that at high solids concentrations the waste could be purified, or was purified, under essentially nonproliferating conditions regardless of the presence or absence of an external nitrogen source. These studies led to separate investigations in which it was shown that substrate could be removed in a nonproliferating phase during which the cells stored carbohydrate, and that this carbohydrate could be subsequently converted to protein in an endogenous stage in which the nitrogen source was added (see Paper 14). The work was conducted in batch, but it led to the proposal of a possible modification to the activated sludge process and to studies which are now under way under continuous flow conditions. Two continuous flow completely mixed activated sludge bench scale plants were first placed in operation for approximately three months. In both units synthetic waste (using glucose as carbon source) was fed to the units without an accompanying source of nitrogen. The mixed liquor then flowed to a settling tank, where the sludge was harvested and channelled to a second aerator which received no raw synthetic waste, but to which a nitrogen source (ammonium ion) was added. Conversion of carbohydrate to protein took place in the second (endogenous) aerator, and the sludge (or a portion thereof) was recycled to the first aerator, where the waste was removed under nonproliferating conditions. In one endogenous aerator, nitrogen was added at a COD:nitrogen ratio of 10:1 based upon the COD of the raw waste and, in the other, nitrogen was added at a COD:nitrogen ratio of 30:1. COD removal in both units was in excess of 95%. The amount of ammonia nitrogen leaking into the effluent from the unit which received a COD:nitrogen ratio of 10:1 in the endogenous phase was approximately 16% of the added nitrogen, whereas no leakage of nitrogen was observed in the effluent from the system which received a COD:nitrogen ratio of 30:1. The sludge produced in both systems exhibited extraordinarily good settleability, and was highly flocculent in character. From these results it seems apparent that if the incoming waste is fairly constant in character and consists of readily-metabolized carbohydrate, this modification could provide protection against the leakage of nitrogen into the receiving stream, thereby helping to control the possibilities for stream enrichment. Work in continuous flow on this system is continuing with particular emphasis on various carbon:nitrogen ratios, and work is also in progress employing other substrates such as acetic acid and lactose.

E. Studies on the Effect of Agitation and Oxygen Tension on the Mechanism and Kinetics of Substrate Removal

The occurrence of, and reasons for, linear sludge production and substrate removal at high biological solids concentrations were discussed in Papers 13 and 15. In no case could linear kinetics be explained on the basis of any physical limitations imposed on the system, such as rate of aeration, or oxygen tension, since 80 to 90% of DO saturation was maintained at all times. However, it has been assumed by some investigators that low oxygen tension is the primary cause for the occurrence of linear kinetics in biological systems. Since oxygen requirements in the activated sludge process are an extremely important concern from a basic as well as an operational and a design standpoint, and since it is generally held that most of the oxygen which is diffused into an activated sludge tank is not used by the organisms but is utilized merely to provide agitation, it was of importance to make some attempt to distinguish between the effect of agitation and the effect of aeration. Studies have been designed and are now in progress to investigate the conditions under which oxygen limitation causes linear kinetics, and also to study the effect of aeration and agitation upon sludge composition. To facilitate these experiments, a reaction vessel fitted with a rotary agitator as well as a means of diffusing air is being employed. Agitation rates of 150, 300, 450, and 600 rpm are being studied. Gas flow rates, in experiments to date, have been held constant at 0.25 l/min/l, and gas streams containing 5, 10, 21, and 100% oxygen have been used. To date preliminary experimentation has provided evidence indicating that there may be a correlation between oxygen supply rate and rate of substrate utilization; however, much more experimentation must be undertaken before such a relationship can be determined. It also has been noted that zero order kinetics can occur, even at dissolved oxygen concentrations as high as 20 ppm.

F. Investigations into the Release of Metabolic Intermediates during Substrate Removal

During the course of investigations on this research project and on others being conducted in the Bioengineering Laboratories, there was evidence that metabolic intermediates or metabolic end products were released during metabolism of the original exogenous substrate. It was possible to detect this phenomenon because multiple analyses for substrate removal were always run. For example, purification rate in all studies was always measured using the COD test for total soluble organic material, and substrate removal was also measured by tests specific for the particular carbon source used in the synthetic waste. If carbon source removal by the specific substrate test was more rapid than by the COD test, the result was taken as evidence for the release of either metabolic intermediates of aerobic metabolism or metabolic end products other than CO_2 and water. Because of the importance of this phenomenon in determining both the overall mechanism and the kinetics of purification, separate studies have been initiated to determine the generality of this occurrence and conditions which would either enhance or prevent the release of such products. In experiments to date the major experimental variables which have been examined for their effect on the release of intermediates are types of substrate and amount of nitrogen in the system. Substrates such as glucose, acetic acid, and sucrose have been studied under growing and under nonproliferating cultural conditions. The release of metabolic intermediates was particularly evident in systems for which acetate was the carbon source, under nonproliferating conditions or under conditions of severe nitrogen deficiency. The studies to date using sucrose are particularly interesting, since metabolic intermediates are released in appreciable quantities and there is a considerable amount of residual COD which does not appear to be removed in a reasonable aeration period.

In these experiments cell composition (protein and carbohydrate content) is being determined for all experiments and in general the results obtained substantiate other results which have been reported from this laboratory concerning the initial synthesis of carbohydrate and its later channelling into protein synthesis. The work is continuing and studies are being conducted to determine the nature of the materials released by the cells during substrate removal. In many of the systems thus far tested there is evidence that at least a portion of the materials released during removal of carbohydrate substrates are free volatile fatty acids.

G. Studies on the Effect of the Degree of Flocculation on Purification Rate

In many of the experiments using a "one pass" continuous flow completely mixed system as well as systems employing cell feedback, there was at times considerable difficulty in obtaining a good settling floc. In the interest of experimental control, it was desirable to determine whether addition of the newer flocculating agents, the polyelectrolytes, could enhance operational characteristics without injuring the biochemical efficiency of the system. It is known that highly dispersed systems usually exhibit a higher biochemical efficiency than do flocculated cells. However, it is necessary that the cells flocculate in order to provide for separation of the mixed liquor under quiescent conditions in the settling tank. Work on the effects of polyelectrolytes on dispersed systems is also important from the mechanistic standpoint, since such work could provide insight into the mechanism of bacterial flocculation, which is an integral part of the overall mechanistic behavior of the activated sludge process. It is emphasized that the operation of the continuous flow units requires considerable expenditure of time and energy, and it was reasoned that, before addition of any flocculating agents, it would be desirable to make a separate study (especially pertaining to the possible toxicity of these agents) rather than hazard the chance of aborting a steady state experiment. Therefore, a research program was initiated pertaining to the efficiency and effect of polyelectrolytes in activated sludge systems. Various anionic and cationic polyelectrolytes were studied in batch systems to determine optimum dosage and to determine the effect of the addition of polyelectrolytes on the biochemical efficiency of the activated sludge system. In all experiments the settling characteristics and the substrate removal patterns were compared with those in control systems which received no polyelectrolytes. It was found that certain dosages of polyelectrolytes (variable, depending upon the polyelectrolyte employed) caused severe retardation of substrate removal. The complete cessation of substrate removal may be interpreted as evidence for the toxicity of the flocculating agents; however, the retardation of the removal may not necessarily indicate toxicity, since the slower removal may come about due to the higher degree of flocculation and limited access of substrate to cells within the floc particles. Experiments are now being conducted in an attempt to determine whether the retardation of substrate removal rate at certain concentrations of polyelectrolytes is due to a toxic effect or is simply due to the fact that cells are existing in a more flocculated community. Some batch experiments are yet to be run; however, most of the work currently under way is being conducted using continuous flow systems.

H. Studies on Analytical Technique and Research Methodology

At various times during the conduct of the research, it has become necessary to make rather exhaustive research efforts pertaining to particular analyses which were needed in the research or to investigate possible modifications in methods which have been used successfully in the biochemical field for specific systems but which may not be directly applicable to systems using heterogeneous populations or media used in the present study. Where these efforts led to modifications which might be of use to other investigators, a separate and expanded study has been performed in order that the method might be published or reported to other investigators for their scrutiny and possible use.

Such was the case for the work which was performed on the colorimetric method for determining chemical oxygen demand (see Paper 1 and/or 1A). In these studies the colorimetric method was shown to yield results equivalent to those obtained by the titrimetric procedure. The colorimetric method represents a time saving device when a large number of COD's must be run, and the rather high cost of an automatic analyzer cannot be justified. The statistical study of the spot plate technique (see Paper 2) for making viable cell counts was needed in order to determine reliable counting limits which could be employed for this method. The method was particularly helpful in studies on the exertion of biochemical oxygen demand in dilute systems. The research methodological work pertaining to the use of chemical oxygen demand values of bacterial cells in waste water purification is particularly helpful in making materials or energy balances for experiments in which data for biological solids, COD removal, and oxygen uptake are obtained. Such balances are necessary as a check on the adequacy of the analytical methods employed. This work is also valuable since it bears on the applicability (or nonapplicability) of empirical formulas depicting the composition of activated sludge.

Other methods which have been investigated for use in the present research and are now being applied include: the dinitrosalicylate method for reducing sugars, the resorcinol method for ketoses, the galactostat test for galactose, and various methods for determining deoxyribonucleic acid, ribonucleic acid, and total nucleic acid. Concerning the various analytical techniques for measuring substrates, it is important to determine the specificity of each method when multisubstrate systems are used or when multisubstrate systems are created due to the release of metabolic intermediates. While the above-mentioned techniques have been investigated and modified for use in the present research, additional investigations are needed (and are under way) to determine the range of conditions under which they may be applied.

LIST OF PUBLICATIONS SUPPORTED WHOLLY OR IN PART BY THE GRANT

1. Gaudy, A. F. Jr., and Ramanathan, M., "A Colorimetric Method for Determining Chemical Oxygen Demand," J. Water Pollution Control Fed., 36, 1479 (1964). Also, Proceedings, 3rd Annual Industrial Water and Waste Conference, Rice University, Houston, Texas, June, 1963.

2. Gaudy, A. F. Jr., and Ramanathan, M., "A Colorimetric Method for Determining Chemical Oxygen Demand," Proceedings, 19th Industrial Waste Conference, Purdue University, 1964, p. 915.

3. Gaudy, A. F. Jr., Abu-Niaaj, F., and Gaudy, E. T., "Statistical Study of the Spot-Plate Technique for Viable Cell Counts," Appl. Microbiol., 11, 305 (1963).

4. Gaudy, A. F. Jr., Bhatla, M. N., and Gaudy, E. T., "The Use of Chemical Oxygen Demand Values of Bacterial Cells in Waste Water Purification," Appl. Microbiol., 12, 254 (1964).

5. Gaudy, A. F. Jr., Bhatla, M. N., and Abu-Niaaj, F., "Studies on the Occurrence of the Plateau in BOD Exertion," Proceedings, 18th Industrial Waste Conference, Purdue University, 1963, p. 183.

6. Follett, R. H., and Gaudy, A. F. Jr., "Studies on the Occurrence of the Plateau in BOD Exertion. III. Simple and Complex Wastes with Various Seed and Substrate Concentrations," Proceedings, 14th Oklahoma Industrial Wastes Conference, Oklahoma Water and Pollution Control Association, 1963, p. 346.

7. Gaudy, A. F. Jr., Bhatla, M. N., Follett, R. H., and Abu-Niaaj, F., "Factors Affecting the Existence of the Plateau during the Exertion of BOD," J. Water Pollution Control Federation, 37, 444 (1965).

8. Bhatla, M. N., and Gaudy, A. F. Jr., "Studies on the Causation of Phasic Oxygen Uptake in High Energy Systems," Proceedings, 19th Industrial Waste Conference, Purdue University, 1964, p. 871. Also J. Water Pollution Control Fed. (in press, 1966).

9. Bhatla, M. N., and Gaudy, A. F. Jr., "Studies on the Plateau in Oxygen Uptake during Exertion of Biochemical Oxygen Demand by Pure Cultures of Bacteria," Biotech. Bioeng., 7, 387 (1965).

10. Bhatla, M. N., and Gaudy, A. F. Jr., "Sequential Substrate Removal in a Dilute System using Heterogeneous Microbial Populations," Appl. Microbiol., 13, 345 (1965).

11. Bhatla, M. N. and Gaudy, A. F. Jr., "The Role of Protozoa in the Diphasic Exertion of BOD," J. San. Eng. Div., Proc. A.S.C.E., 91, SA3 (4371), 63 (1965).

12. Rao, B. S., and Gaudy, A. F. Jr., "Effect of Sludge Concentration on Various Aspects of Biological Activity in Activated Sludge," J. Water Pollution Control Fed., 38, 794, (1966). Also, Proceedings, 5th Industrial Waste Conference, Texas Water Pollution Control Association, 1965, p. 216.

13. Komolrit, K., Goel, K. C., and Gaudy, A. F. Jr., "Regulation of Exogenous Nitrogen Supply and its Possible Application to the Activated Sludge Process," presented at the 38th Annual Conference, Water Pollution Control Federation, Atlantic City, N. J., October, 1965. Also J. Water Pollution Control Fed. (in press, 1966).

14. Krishnan, P., and Gaudy, A. F. Jr., "Mechanism and Kinetics of Substrate Utilization at High Biological Solids Concentrations," Proceedings, 21st Industrial Waste Conference, Purdue University, 1966 (in press).

15. Gaudy, A. F. Jr., Ramanathan, M., and Rao, B. S., "Kinetic Behavior of Heterogeneous Populations in Completely Mixed Reactors," scheduled for presentation at the National Meeting, American Chemical Society, New York, September, 1966, and for publication in Biotechnology and Bioengineering.

PROFESSIONAL PERSONNEL

Dr. Anthony F. Gaudy, Jr. is the principal investigator and has devoted from 25 to 40% of his research effort to the accomplishment of this research.

Dr. Elizabeth T. Gaudy, Associate Professor of Microbiology, Microbiology Department, is not employed on the project, but has devoted some research effort to the conduct of the work (see publications #2 and #3).

Dr. Gaudy has been assisted in the conduct of the work by a number of graduate students who are listed below.

M. N. Bhatla

M. N. Bhatla began his doctoral program in September, 1961, and was associated with this project from June, 1963, to September, 1964, at which time he completed his work. He was formally awarded the Ph.D. degree in May, 1965. The title of his thesis is: "Studies on the Kinetics and Mechanism of Phasic Oxygen Uptake with Special Regard to the BOD Test."

M. Ramanathan

M. Ramanathan began his doctoral program in February, 1962, and was associated with the project from September, 1962, to June, 1966, at which time he completed his work.

He was formally awarded the Ph.D. degree in August, 1966. The title of his thesis is: "Kinetics of Completely-Mixed Activated Sludge."

R. H. Follett

R. H. Follett began his master's program in June, 1962. He was associated with the work of the project (but received no salary from the project) from September, 1962, to August, 1963, at which time he received his master's degree. The title of his thesis is: "Studies on the Diphasic Nature of BOD Progression."

F. N. Abu-Niaaj

F. N. Abu-Niaaj began his master's program in September, 1961. He was associated with research allied to this project and participated in some of the preliminary research which was accomplished prior to initiating the project. He completed his work and received the master's degree in August, 1962. The title of his master's report is: "Study of the Velocity of Expression of Biochemical Oxygen Demand."

B. S. Rao

B. S. Rao began his master's program in January, 1963, and was associated with this project until September, 1964. He received his master's degree in August, 1964. The title of his thesis is: "Effect of Initial Biological Solids Concentration on Biochemical Response in Discontinuous Activated Sludge Systems."

K. C. Goel

K. C. Goel began his doctoral program in September, 1964, and has been associated with the project since that time.

G. Thabaraj

G. Thabaraj began his doctoral program in January, 1965, and has been associated with the project since that time.

M. D. Rickard

M. D. Rickard began his doctoral program in June, 1965. His research is associated with the project research; he does not receive any salary from the project.

M. L. Woldman

M. L. Woldman began his master's program in September, 1965, and has been associated with the project since that time.

T. D. Yu

T. D. Yu began his master's program in September, 1964. He accomplished his master's research in an area related to this project and received his degree in May, 1966. He received no salary from the project. The title of his master's thesis is: "Effects of Polyelectrolyte Coagulant Aids on Bacterial Cells."

ABSTRACTS OF PAPERS AND PUBLICATIONS
OF RESEARCH SUPPORTED BY
GRANT WP-00325

Paper 1

Gaudy, A. F. Jr., and Ramanathan, M., "A Colorimetric Method for Determining Chemical Oxygen Demand," Journal Water Pollution Control Federation, 36, 1479 (1964). Also Proceedings, 3rd Annual Industrial Water and Waste Conference, Rice University, Houston, Texas, June, 1963.

This study, comparing results of COD determinations by the standard titrimetric procedure and by colorimetry, was made using a variety of waste waters. The results indicated that the two procedures yield comparable COD values. The correlation between these methods was very good when the turbidity of the reflux sample was low. Addition of the catalyst at the beginning of the reflux period leads to precipitation of silver chloride in samples containing high chloride concentrations. An alternate procedure wherein the sample was refluxed in the absence of $AgSO_4$ followed by refluxing in its presence was shown to avoid the formation of a precipitate.

A copy of this paper is not included, since Paper 1A contains the same information plus additional data.

Paper 1A

Gaudy, A. F. Jr., and Ramanathan, M., "A Colorimetric Method for Determining Chemical Oxygen Demand," Proceedings, 19th Industrial Waste Conference, Purdue University, 1964, p. 915.

This paper was presented under the same title as Paper 1, but included additional results in which the work was extended to cover a wider variety of waste waters. The paper was presented at the Purdue Industrial Waste Conference at the specific request of Professor Bloodgood.

Paper 2

Gaudy, A. F. Jr., Abu-Niaaj, F., and Gaudy, E. T., "Statistical Study of the Spot-plate Technique for Viable Cell Counts," Appl. Microbiol., 11, 305 (1963).

A statistical study of the spot-plate technique was made for the purpose of establishing an acceptable counting range. The organism used in these studies was *Micrococcus lysodeikticus*. Standard deviations and coefficients of variation were computed for counts ranging from 20 to 440. The lower and upper limits for acceptable counts, based on coefficients of variation of 10 and 5.8, respectively, were chosen as 100 and 300.

Paper 3

Gaudy, A. F. Jr., Bhatla, M. N., and Gaudy, E. T., "The Use of Chemical Oxygen Demand Values of Bacterial Cells in Waste Water Purification," Appl. Microbiol., 12, 254 (1964).

Four methods for determining substrate recoveries in studies concerned with the partition of substrate between sludge synthesis and respiration were investigated. An energy balance, comparing chemical oxygen demand (COD) removed with the summation of oxygen uptake and the COD of the cells produced, yielded average recoveries closer to 100 per cent than any of the other three methods tested. The standard COD test was shown to yield highly reproducible values when used to determine the COD of activated sludge. Although the protein and carbohydrate content of the cells varied with cell age, a concomitant variation in cell COD was not noted.

Paper 4

Gaudy, A. F. Jr., Bhatla, M. N., and Abu-Niaaj, F., "Studies on the Occurrence of the Plateau in BOD Exertion. I. Heterogeneous and Pure Culture Seeds with Various Concentrations of Glucose as Carbon Source." (Portions of this paper were presented at the 18th Industrial Waste Conference, Purdue University, 1963.)

There had been an increasing number of conflicting reports concerning the occurrence of a plateau in oxygen uptake during the exertion of biochemical oxygen demand. This mechanistic aspect of substrate removal is of great significance in determining the kinetics of waste water purification by activated sludge, and is also important from the standpoint of analyzing the results of the BOD test. It was essential to determine the conditions under which the plateau could be manifested, and hopefully to gain some insight into the causative mechanisms for its occurrence. The studies herein reported were undertaken primarily to determine the generality or constancy of the occurrence of the plateau with various seeding conditions and substrate concentrations in the BOD bottle. In these studies the carbon source consisted of glucose. The results showed with reasonable certainty that the plateau in oxygen uptake during exertion of carbonaceous BOD occurs using various seeding materials and glucose as the carbon source. Analysis of the oxygen uptake data and bacterial count data taken at each sampling point for DO provided a clear indication that the rapid rise in oxygen uptake (first stage) corresponded to rapid bacterial multiplication. The plateau corresponded to the peak range of population density (the stationary phase); the second stage of O_2 uptake corresponded to a decreasing portion of the bacterial count curve.

Paper 5

Gaudy, A. F. Jr., and Bhatla, M. N., "Studies on the Occurrence of the Plateau in BOD Exertion. II. Experiments using Different Carbohydrates and Multicomponent Systems." (Portions of this paper were presented at the 18th Industrial Waste Conference, Purdue University, May, 1963.)

The results presented herein represent an extension of the work reported in Paper 4. In these studies the work was extended to include a variety of compounds using young heterogeneous populations acclimated to the particular compound under study, and to multicomponent substrate systems using both young heterogeneous populations and a pure culture seed. The substrates employed included glycerol, glycogen, lactose, ribose, glutamate, sorbitol, melibiose, galactose, and various combinations of these compounds with glucose. The work indicated that the plateau could occur with a variety of carbon sources, and that the duration or severity of the plateau corresponded approximately to the length of the stationary phase and the region of maximum population. Based upon these studies, four related theories which could possibly explain the occurrence of the plateau were formulated.

Paper 6

Gaudy, A. F. Jr., Bhatla, M. N., and Abu-Niaaj, F., "Studies on the Occurrence of the Plateau in BOD Exertion," Proc. Industrial Waste Conference, Purdue University, XLVIII, 183, May, 1963.

Portions of the results presented in Papers 4 and 5 are combined in this paper.

Paper 7

Follett, R. H., and Gaudy, A. F. Jr., "Studies on the Occurrence of the Plateau in BOD Exertion. III. Simple and Complex Wastes with Various Seed and Substrate Concentrations," Proceedings, 14th Oklahoma Industrial Wastes Conference, Oklahoma Water and Pollution Control Association, 1963, p. 346.

This paper represents an extension of the work reported in Papers 4 and 5, and includes investigations of the effects of substrate concentration and the size of the initial seed inoculum on the occurrence of the plateau in BOD exertion. Also included were studies on the occurrence of the plateau using complex media and whole wastes. Phasic oxygen uptake in the BOD bottle occurred in all systems tested; however, the plateau between phases was not evidenced for nutrient broth.

Paper 8

Gaudy, A. F. Jr., Bhatla, M. N., Follett, R. H., and Abu-Niaaj, F., "Factors Affecting the Existence of the Plateau during the Exertion of BOD," J. Water Pollution Control Fed., 37, 444 (1965).

In this paper the results reported in Papers 4, 5, and 7 were summarized for journal publication. In summary, at this point of the investigation it was concluded that the occurrence of the plateau in BOD exertion appeared to be independent of the type of seed employed. The plateau was exhibited over a wide range of substrates. It occurred in both single carbon source systems and in systems containing a complex mixture of organic compounds. Within the usual limits of seed concentration employed in the BOD test, the occurrence of the plateau was not dependent upon seed concentration. The relation between oxygen uptake and viable bacterial count remained consistent throughout the investigation, and four theories of causation for the occurrence of the plateau were derived, based upon evidence thus far obtained.

Paper 9

Bhatla, M. N., and Gaudy, A. F. Jr., "Studies on the Causation of Phasic Oxygen Uptake in High Energy Systems," Proceedings, 19th Industrial Waste Conference, Purdue University, 1964, p. 871 (J. Water Pollution Control Fed., in press, 1966).

Studies were undertaken using heterogeneous populations developed on a glucose-salts medium from an initial sewage seed for the purpose of examining possible causative mechanisms for the occurrence of the plateau in BOD exertion. In these studies substrate and cell concentrations much greater than those employed in the BOD bottle were used. The studies included investigations on the effect of substrate concentration and initial solids concentration on the occurrence of the plateau using both sewage seed and acclimated seed. In addition, long term aeration studies were made to determine possible relationships between phases of oxygen uptake and substrate removal, intracellular protein content, and solids production. Efforts were also made to determine whether carbon sources other than the original carbon source were excreted into the medium during the plateau or the second stage of oxygen uptake. It was found for substrate concentrations up to 1000 mg/l that there was no distinct or critical substrate concentration at which the plateau could not be observed. The results did indicate, however, that the phases gradually merge, and that the plateau tends to become indistinct and may finally disappear as the total energy of the system increases. From these studies it appeared that the release of cellular components due to lysis of a portion of the bacterial population after reaching the maximum attainable numbers and the need for an acclimation period before metabolism of these released components could not be cited as a general causative mechanism for a plateau in BOD exertion by heterogeneous populations. Also, in these particular studies it was found that the release of intermediates or end products during the metabolism of the original exogenous substrate could not be cited as a causative mechanism contributing to the existence of a plateau for heterogeneous populations.

Paper 10

Bhatla, M. N., and Gaudy, A. F. Jr., "Studies on the Plateau in Oxygen Uptake during Exertion of Biochemical Oxygen Demand by Pure Cultures of Bacteria," Biotech. and Bioeng., VII, 387 (1965).

Evidence for generation of the plateau in oxygen uptake during exertion of carbonaceous BOD by pure cultures was sought by making long-term Warburg studies using glucose and phthalic acid as substrates. One organism, tentatively identified as *Escherichia intermedia*, of the fifty-seven tested, exhibited a plateau. Detailed studies on this organism indicated that generation of the plateau depended upon the type of substrate and, to some extent, upon the initial cell concentration employed, but not upon substrate concentration. Oxygen utilization during the second stage was accompanied by metabolism of secondary extracellular carbon source(s) produced by these cells during metabolism of the original extracellular carbon source (glucose) in the first phase of O_2 uptake. The secondary substrate was registered as volatile acids. Selective inhibition of protein synthesis at various times during oxygen uptake led to the conclusion that the plateau occurred because of the need for an induction period before the secondary substrate could be metabolized. Using this organism, it was also found that a plateau could be generated during endogenous respiration. This secondary oxygen utilization was accompanied by uptake of organic materials excreted into the medium by the cells during metabolism of endogenous carbon sources.

Paper 11

Bhatla, M. N., and A. F. Gaudy, Jr., "Sequential Substrate Removal in a Dilute System using Heterogeneous Microbial Populations," Appl. Microbiol., 13, 345 (1965).

The sequential metabolism of substrates by heterogeneous bacterial populations has been previously reported from this laboratory in studies using high substrate concentrations. This phenomenon has now been shown to occur at very dilute substrate concentrations, i.e., 5 mg/l glucose + 5 mg/l sorbitol, in studies conducted under the conditions of the standard biochemical oxygen demand (BOD) test. Sequential metabolism of these substrates resulted in a diphasic curve of accumulated oxygen uptake, wherein the two phases were separated by a discernible plateau. These findings illustrate one possible explanation for the generation of discontinuity in the kinetic course of carbonaceous BOD exertion.

Paper 12

Bhatla, M. N., and Gaudy, A. F. Jr., "The Role of Protozoa in the Diphasic Exertion of BOD," *Journal Sanitary Engineering Division*, *Proceedings* *ASCE*, *91*, SA3 (4371), 63 (1965).

Causation for the occurrence of a plateau in diphasic BOD curves was investigated for heterogeneous seeds of sewage origin. Oxygen uptake studies were conducted in discontinuous systems using glucose synthetic waste, employing manometric techniques as well as standard BOD dilution procedure. Bacterial and protozoan population growth patterns were followed during the course of oxygen utilization. The effect of the protozoa-bacteria relationship on the kinetics of oxygen uptake and the occurrence of the plateau was examined. In addition, the effect of inoculations of protozoa to bacterial systems was studied with respect to oxygen demand and other biochemical parameters. Pasteurization and microfiltration of the seed material were employed to exclude protozoa from the bacterial population in some studies. Chloramphenicol was used to delineate the role of bacterial enzyme synthesis in generating the plateau and the second stage of oxygen uptake.

It was found that for heterogeneous populations the second stage of oxygen uptake was due almost entirely to protozoan metabolism, and that the length of the plateau was related to the relative numbers of bacteria and protozoa in the seed material. When the plateau occurred, it corresponded to the range of maximum viable bacterial count, and the secondary rise in oxygen uptake was marked by rapid multiplication of the protozoa; approximately fifty per cent of the second stage oxygen uptake had been exerted when the protozoan population reached maximum density. It was found that for heterogeneous populations a two-phase oxygen uptake curve during exertion of carbonaceous BOD is caused by the sequential growth of two segments of the population, i.e., the growth of bacteria, removing the exogenous carbon source, and the growth of the protozoa, using the bacteria produced in the first stage as food. In contrast to prevalent concepts in the field, the protozoa were found to play a direct and autonomous role in the exertion of carbonaceous biochemical oxygen demand.

Paper 13

Rao, B. S., and Gaudy, A. F. Jr., "Effect of Sludge Concentration on Various Aspects of Biological Activity in Activated Sludge, J. Water Pollution Control Fed., 38, 794 (1966); also Proceedings, 5th Industrial Waste Conference, Texas Water Pollution Control Association, 1965, p. 216.

There have been various and somewhat conflicting reports in the literature concerning the relationship between sludge concentration and substrate removal rate for activated sludge systems. It has been reported that a simple linear relationship between the rate of substrate removal and initial sludge concentration exists, and that the relation is dependent upon the type of carbon source or type of waste being metabolized. The implication was that the slope of the linear relationship was specific for a particular type of waste. Also, there have been many conflicting reports pertaining to the sludge yield. Here again the implication is that the sludge yield is determined by the type of substrate or waste or its free energy content. Although it would be ideal from an engineering standpoint if such relationships were entirely substrate-dependent, there were no published data to show that the relationship remains constant over a prolonged period of operation. It was felt that a considerable insight into the constancy of the cell yield and the relation between substrate removal rate and initial solids concentration could be obtained by conducting an integrated long-term study under highly controlled operational conditions using only one carbon source. Both aspects have an important bearing on the kinetics and mechanism of waste water purification by the activated sludge process. The long-term studies indicated that there is a straight line relationship between initial solids concentration and substrate removal rate for a specific sludge. However, for sludge samples taken at different times from a unit operated over a long period, the following observations were made:

1. The relationship between initial solids concentration and rate of COD removal varies for a single substrate.

2. The cell yield varies for a single substrate.

3. The percentage of the theoretical oxygen demand exerted at the point of both glucose and COD removal decreases as initial solids concentration increases.

The variations in the relationship between initial biological solids concentration and purification rate and in cell yield appear to be due to changes in predominance in the population which occur in spite of controlled, constant operational conditions. The decrease in percentage theoretical oxygen demand exerted at substrate removal when initial solids are increased can be explained on the basis of differences in the amount of the total substrate utilized in early synthesis of carbohydrates. This synthesis, which occurs when starved cells are placed in fresh medium, requires less energy and therefore consumes less oxygen than do synthetic processes which take place after the cells have become adjusted to the new environment.

Paper 14

Komolrit, K., Goel, K. C., and Gaudy, A. F. Jr., "Regulation of Exogenous Nitrogen Supply and Its Possible Application to the Activated Sludge Process," presented at the 38th Annual Conference of the Water Pollution Control Federation, Atlantic City, October, 1965. In press, J. Water Pollution Control Fed., 1966.

Work was undertaken to determine the effect of controlled periodic addition of nitrogen on the treatment of nitrogen-deficient synthetic waste by activated sludge. Batch activated sludges were developed (24-hour feeding cycle; 1/3 sludge wasting), using glucose as carbon source and ammonium sulfate as the source of nitrogen. After a period of balanced operation, the organic loading was continued but nitrogen was withheld. After several such feeding periods during which no sludge was wasted, the sludge was parted and its metabolic characteristics were studied under endogenous conditions, with addition and without addition of nitrogen source. These sludges were then washed and subjected to substrate loadings in the presence and absence of a nitrogen source. During all phases of study the partition between respiration and synthesis was compared in the presence and the absence of nitrogen. The parameters investigated included COD removal, glucose removal, oxygen uptake, and biological solids production. Sludge composition (carbohydrate and protein) was also determined in all experiments to gain information on the biochemical nature of the sludge and changes occurring under the various operational conditions studied.

It was found that the sludge retained an ability to remove the carbon source for some time in the absence of an exogenous nitrogen source when the loading was applied at 24-hour intervals. However, the activity of the sludge, as measured by the COD removal rate and respiration rate, gradually decreased as the cell age increased. In the absence of an exogenous nitrogen source, the substrate was channelled mainly into carbohydrate synthesis. The decrease in sludge activity was related to an accumulation of synthesized carbohydrate and a decrease in protein content as the sludge age increased. However, the activity of this high carbohydrate-containing sludge could be considerably restored upon addition of nitrogen under endogenous conditions. The addition of the nitrogen source also resulted in a significant increase in the endogenous respiration rate of the sludge, and in the internal conversion of the stored carbohydrate to protein. The increased activity accompanying this change in chemical composition of the sludge was manifested by a significant recovery in substrate removal rate and respiration rate upon re-feeding with organic waste in the absence of an exogenous source of nitrogen.

The results of the study suggest that the activity of activated sludge is governed largely by its protein content, and that the nitrogen requirement of activated sludge may be supplied intermittently or only in the endogenous stage rather than simultaneously with the organic loading. A possible flow sheet incorporating aspects of biosorption and extended aeration was presented. Such operation may result in considerable savings in nitrogen and decreased opportunity for mineral nutrition problems in the receiving stream.

Paper 15

Krishnan, P., and Gaudy, A. F. Jr., "Mechanism and Kinetics of Substrate Utilization at High Biological Solids Concentrations," Proceedings 21st Industrial Waste Conference Purdue University, May, 1966 (in press).

In a previous paper Rao and Gaudy (see paper 13) presented a theory based upon experimental findings which explained why the percentage of the theoretical oxygen demand exerted at the time of substrate removal should decrease as initial biological solids concentration was increased. However, the highest biological solids concentration employed in the previous work was approximately 2000 mg/l, and it was felt that further studies at higher solids concentrations were warranted. In addition, work at higher solids concentrations permitted investigations into the amount of substrate which could be immediately taken up by the sludge. Since very rapid purification is accomplished only at high solids concentrations, the present study offered an opportunity to gain further insight into the possible roles of adsorption and biological assimilation during rapid substrate removal. The work is particularly pertinent to the biosorption process, and to the applicability of a process modification recently reported by Komolrit, Goel, and Gaudy (see Paper 14). It was found that the sludge yield for initial solids concentrations up to 5000 mg/l was in the general range of those previously found at 2000 mg/l and below. Concerning the biochemical mechanism of substrate removal, the work which formed the basis of the theory presented by Rao and Gaudy at lower solids concentrations was substantiated at the high solids concentrations used in the present study. When the initial solids level was high, almost all of the substrate could be removed before an appreciable amount of protein was synthesized. It is to be expected that even when starting with low initial biological solids concentrations, carbohydrate synthesis precedes protein synthesis; however, there are so few cells in the system that the early synthesis of carbohydrate does not result in appreciable substrate removal. In this case, substrate removal actually does depend on balanced growth, i.e., replication of the cells; whereas in high solids systems it does not. Therefore, the solids level affects the mechanism by which the substrate is removed. Under conditions of high solids, where little or no protein is made during the period of substrate removal, it would be expected that purification would not be dependent upon a source of nitrogen. The ability of high solids systems to remove substrate in the complete absence of protein synthesis was rigorously demonstrated by inhibiting protein synthesis with chloramphenicol. The experimental data demonstrating the initial conversion of substrate to carbohydrate also made it possible to offer a sound biochemical explanation for the approximately zero order kinetics of substrate and COD removal, oxygen uptake, and solids production often observed with high solids systems. Since these systems function initially as nonproliferating systems, they would be expected to exhibit kinetics typical of such systems, i.e., linear or zero-order kinetics. The results of experiments using sorbitol as substrate throw particular light on the question of adsorption as a primary mechanism of substrate removal. Sorbitol does not react to the anthrone test, and therefore it does not register as carbohydrate. However, during substrate removal, the carbohydrate content of the sludge increased rather rapidly. This finding indicates that the removal of sorbitol was the result of its biochemical conversion into carbohydrate, thus providing evidence against the adsorption mechanism.

Paper 16

Gaudy, A. F. Jr., Ramanathan, M., and Rao, B. S., "Kinetic Behavior of Heterogeneous Populations in Completely Mixed Reactors," to be presented at the National Meeting, American Chemical Society, New York, September, 1966.

Long term investigations of the kinetic behavior of heterogeneous microbial populations were undertaken in continuous flow completely mixed reactors operated at various dilution rates. Glucose was used as the growth-limiting nutrient. The physiological growth parameters for cells harvested from continuous flow reactors were determined using batch experiments. It was found that the growth parameters, maximum growth rate (μ_m), saturation constant (k_s), and cell yield (Y), varied for each dilution rate, and cannot be considered as precise constants in depicting the kinetic behavior of heterogeneous populations. In addition, it was found that the yield coefficients obtained from batch experiments were always lower than those obtained from continuous flow experiments. Levels of substrate and biological solids calculated for different dilution rates using growth constants from batch experiments did not agree with the experimental values observed in steady state experiments. However, when the yield values from the continuous flow experiments were used in conjunction with batch values for μ_m and k_s, the theoretical and experimental dilute-out curves agreed fairly closely (within the range needed for engineering prediction) until the culture began to wash out of the unit. In general, the data substantiated the use of the single phase relationship between growth rate and substrate concentration described by the Monod equation,

$$u = \frac{\mu_m S}{k_s + S} .$$

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PAPER 1A

A COLORIMETRIC METHOD FOR DETERMINING CHEMICAL OXYGEN DEMAND

By

A. F. Gaudy, Jr. and M. Ramanathan

Presented at the 19th Industrial Waste Conference, Purdue University, May, 1964. This paper has the same title and contains the same information as Paper 1, plus additional information on application of the method to a wider variety of waste waters. For this reason, Paper 1 is not included. Presentation of this paper at the Purdue conference was specifically requested by Professor Bloodgood.

A COLORIMETRIC METHOD FOR DETERMINING CHEMICAL OXYGEN DEMAND

By

A. F. Gaudy, Jr.,[1] and M. Ramanathan,[2]
Oklahoma State University, Stillwater, Oklahoma

In June, 1963, at the Texas Industrial Waste Conference, the authors presented the results of their research on a colorimetric COD technique.(1) Since the paper proved to be of interest to many workers, we were delighted when Professor Bloodgood asked us to present these results at the Purdue Industrial Waste Conference. During the past year we have extended the work to include a wider variety of waste waters. The present report includes some of the new work as well as the results previously reported.

Although the chemical oxygen demand (COD) test may not, under certain circumstances, be directly correlated with the established biochemical oxygen demand (BOD) test, it has been increasingly used for estimating the pollutional potential of waste waters and the efficiency of biological waste treatment systems. Today, even in cases where major reliance is placed upon the BOD test, waste characterization usually includes both COD and BOD data. In some cases greater reliance is placed upon the results of the COD test. Its chief advantage over the BOD determination is the speed with which results

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Presented at the 19th Purdue Industrial Waste Conference, Purdue University, Lafayette, Indiana, May, 1964.

are obtained, thereby suggesting its use as a valuable parameter for controlling biological treatment plant operation. Regardless of the speed and relative ease with which the determination is performed, it is nevertheless necessary to determine the normality of the ferrous ammonium sulfate titrant daily, and to prepare and titrate a blank regardless of the number of analyses made. When the number of samples is large, the titration procedure itself becomes laborious and time-consuming. These considerations and the lack of qualified manpower at some treatment plants discourage greater use of the COD test in plant operation. These and other aspects which militate against more adequate laboratory control, especially in the small treatment plant, have been delineated by Forman and Annett.(2)

Current investigations in our laboratory make use of the COD test as one of the parameters for assessing system behavior. At times up to 75 COD determinations may be made in one day, and efforts have been made to devise a more rapid analytical procedure. Many investigators have reported short term tests in which the reflux time is reduced. While for many of the substrates used in our present investigations a reflux time shorter than the standard 2-hour period could be used, it was felt that the general application of any time-saving procedure would be lessened by deviating from this requirement of the standard test. Accordingly it was decided to attempt a colorimetric determination, thus obviating the need for titration. It was reasoned that a colorimetric procedure would also be of general value since it might provide treatment plant personnel with an easier, less time-consuming method, thus encouraging more complete laboratory control at the plant.

Colorimetric tests for organic matter which depend upon the oxidizing capabilities of dichromate in acid solution have been applied for some time in areas other than the sanitary field. Johnson has described a rapid method for determination of nonvolatile organic material.(3) In this method dichromate is used as an oxidant and determination of excess dichromate may be made colorimetrically or by titration. The method is very similar to that of Bloor for lipid analysis.(4) Johnson's colorimetric method requires preparation of two blanks, one in which no dichromate is reduced (similar to the blank prepared in the standard COD test), and one in which all dichromate is reduced by the addition of an excess of Na_2SO_3. The reduced blank is set at 100% transmittance at 440 mμ, and the remaining tubes read against it. A boiling period of twenty minutes is required. A somewhat similar method is reported by Neish.(5) However, in this method the oxidized blank is set at 100% transmittance at 650 mμ, and all samples read against it. As with the method reported by Johnson, another blank is prepared and treated with sufficient bisulfite to reduce all the dichromate. This blank gives the percent transmittance for a known concentration of trivalent chromium. The method depends upon the production of the green color of trivalent chromium ion, in accordance with the Beer-Lambert law, as a result of dichromate reduction. Another method employing only the oxidized blank with tubes read against it at 600 mμ has also been used.(6)

In the industrial waste field, McNary, Dougherty and Wolford have used a colorimetric method for determining the COD of citrus wastes.(7) They were concerned with reducing the time required to obtain COD data. This was successfully accomplished for their particular waste by reducing the reflux time and by using colorimetric measurements. Absorption spectra were obtained for both oxidized and reduced dichromate. The reduced dichromate spectrum showed two minima in percent transmittance, one at approximately 440 and another at 600 mμ. However, it was decided to use a wave length of 650 mμ since this wave length yielded the greatest difference in percent transmittance between oxidized and reduced blanks (52%). It was reasoned that at this wave length there would be less interference between the color of the reduced blank and that of the oxidized blank. COD was calculated by comparison with a standard curve prepared using sucrose. In general the colorimetric procedure was found to be satisfactory. Colorimetric analysis in the determination of chemical oxygen demand is also used in an automated device wherein samples are read at 440 mμ.(8)

From the above discussion it is seen that there are various colorimetric methods for measuring the chemical oxygen demand of organic matter. However, it is noted that there is a variety of procedures which may be followed. Also there is very little information in the literature pertaining to the general applicability of a colorimetric procedure to various waste waters. In the work herein presented the aim was simply to determine whether COD values obtained by the standard titrimetric procedure were equivalent to those obtained colorimetrically when identical samples were subjected to identical reflux conditions. Accordingly work was undertaken to determine the equivalence of the two procedures using municipal, indistrial, and joint wastes as well as a standard compound.

MATERIALS AND METHODS

In all, nine sets of waste samples were examined in replicate experiments. These included a synthetic waste in which glucose was the sole source of carbon, raw sewage and primary effluent, both from the Stillwater municipal waste treatment plant, a refinery waste, primary effluent and activated sludge mixed liquor, both from the North side plant in Oklahoma City, Oklahoma, and digester liquor (1/25 dilution), white water (1/10 dilution), and plant effluent from a hardboard plant. All experiments were carried out in duplicate, and for all wastes examined COD determinations were made with and without addition of the silver sulfate catalyst. Samples were treated in accordance with Standard Methods, using the two-hour reflux period.(9) The colorimeters used in these studies were the Bausch and Lomb " Spectronic 20", and Coleman Model 6D. While more sensitive instruments would be desirable for research applications, it was felt that the general applicability of the technique should be tested using instruments commonly found in smaller municipal and industrial waste treatment plants.

It was also felt, in the interest of simplicity, that the method should not require preparation of a totally reduced blank. Accordingly the technique which was tested provided for the preparation and refluxing of the standard (totally oxidized) reagent blank. The wave length setting used was based upon the maximum absorption of partially reduced dichromate solution (green color of trivalent chromium) using the oxidized blank for setting the instrument at 100% transmittance. It is emphasized that during these experiments no attempt was made to alter volumes or normalities of reagents prescribed in Standard Methods. The only difference between the colorimetric method and the standard titration method involved determination of the optical density of the sample after refluxing instead of performing the usual titration.

RESULTS AND DISCUSSION

Optimum Wave Length

After refluxing reagents blanks and various samples containing organic matter, the optimum wave length was determined using the difference spectrum obtained by setting the instrument to 100% transmittance with the reagent blank prior to each measurement of percent transmittance at various wave length settings. It is seen in Figure 1 that the maximum absorption took place at 600 mμ. These tests were repeated at different concentrations, with different types of organic matter using the "Spectronic 20", and were also checked with a Coleman 6D colorimeter and a Beckman DU spectrophotometer. Selection of this wave length as the optimum when the instrument is set at zero with the oxidized blank is not based upon the spectrum for either completely oxidized (orange) or completely reduced (green) dichromate, but upon the presence of both colors, which is in fact the condition under which the COD test is run.

Relation between Optical Density and COD

Glucose was chosen as the standard organic compound since its observed COD is very close to its theoretical value; that is, it is totally oxidized under the conditions of the test and thereby allows

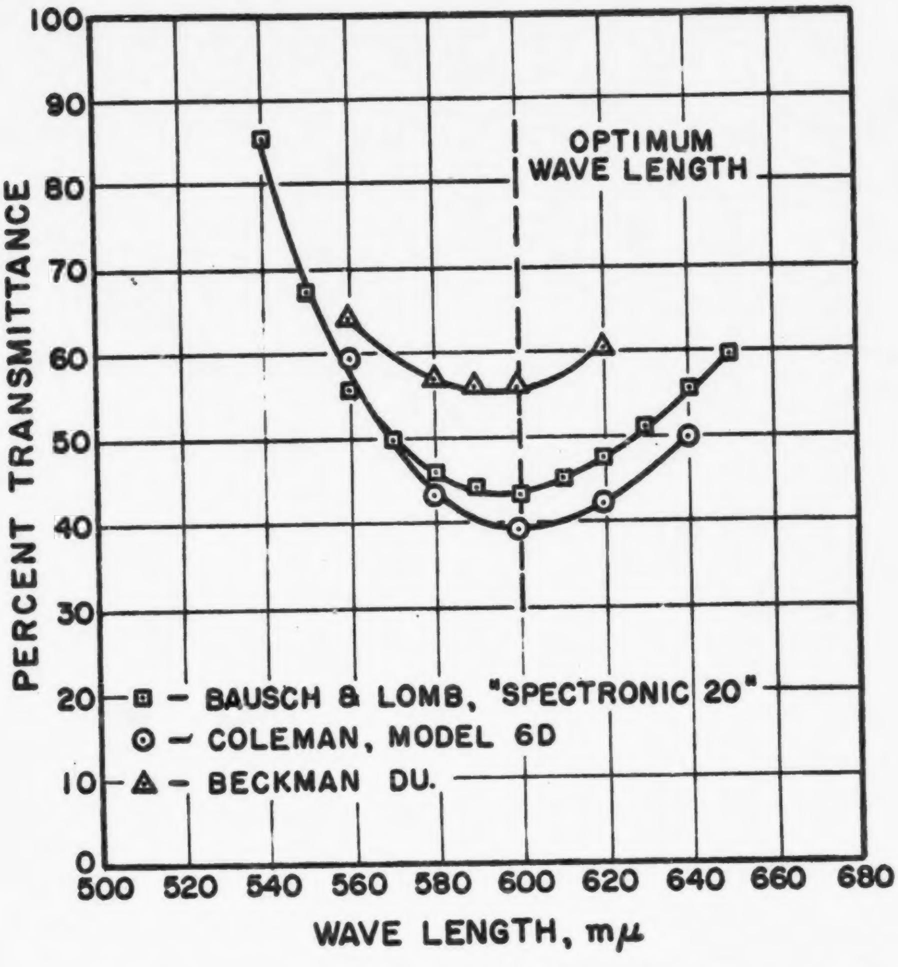

Fig. 1 – Percent Transmittance Vs. Wave Length for Colorimetric COD Method.

direct comparison of optical density with either observed COD or weight of organic matter.

From a standard solution containing 1 gm of glucose per liter, various dilutions were prepared in quadruplicate and COD analyses made. Duplicates of each concentration were taken for titration and for colorimetric analysis. In Figure 2 optical density is plotted against the COD obtained by titration for samples refluxed with and without silver sulfate. The Beer-Lambert law is followed and the line of best fit yields the relationship, optical density = 0.0087 mg COD, with the "Spectronic 20", and 0.00834 mg COD with the "Coleman 6D".

From this result it was apparent that a colorimetric procedure could be used in place of the titration. Similar tests were run using the whole wastes previously listed. Typical results for a refinery waste are shown in Figure 3. The observed slope agrees closely with that shown for glucose. The data for all types of organic-containing waters tested using the "Spectronic 20" are shown in Figure 4 for COD's refluxed without the silver sulfate catalyst. The line of best fit through these data yields a slope of 0.0088. This value compares favorably with that found for the glucose standard; therefore the standard glucose curve accurately depicts the relationship between optical density and the COD value obtained by titration for all wastes tested.

When the catalyst was added at the beginning of the reflux period the relationship was not as constant, as shown in Figure 5. For glucose and the refinery effluent the correlation between optical

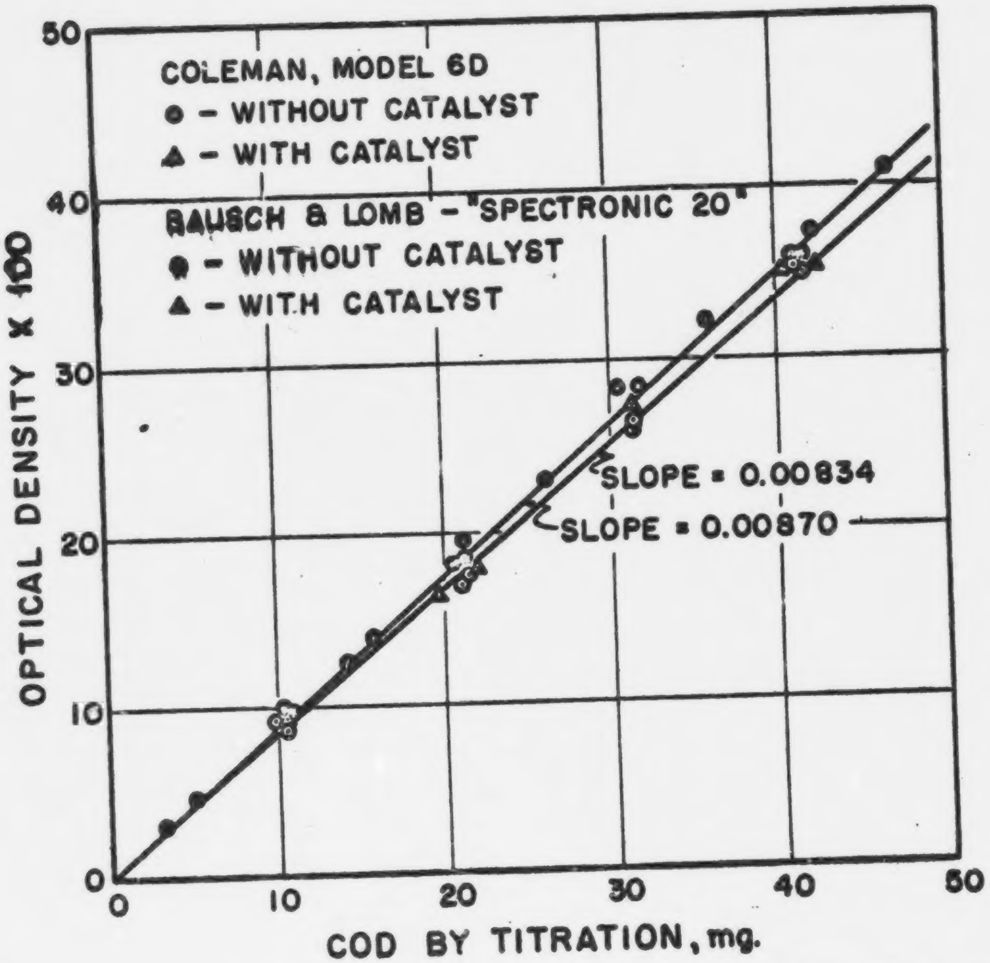

Fig. 2–Relationship Between Optical Density and COD, for Glucose.

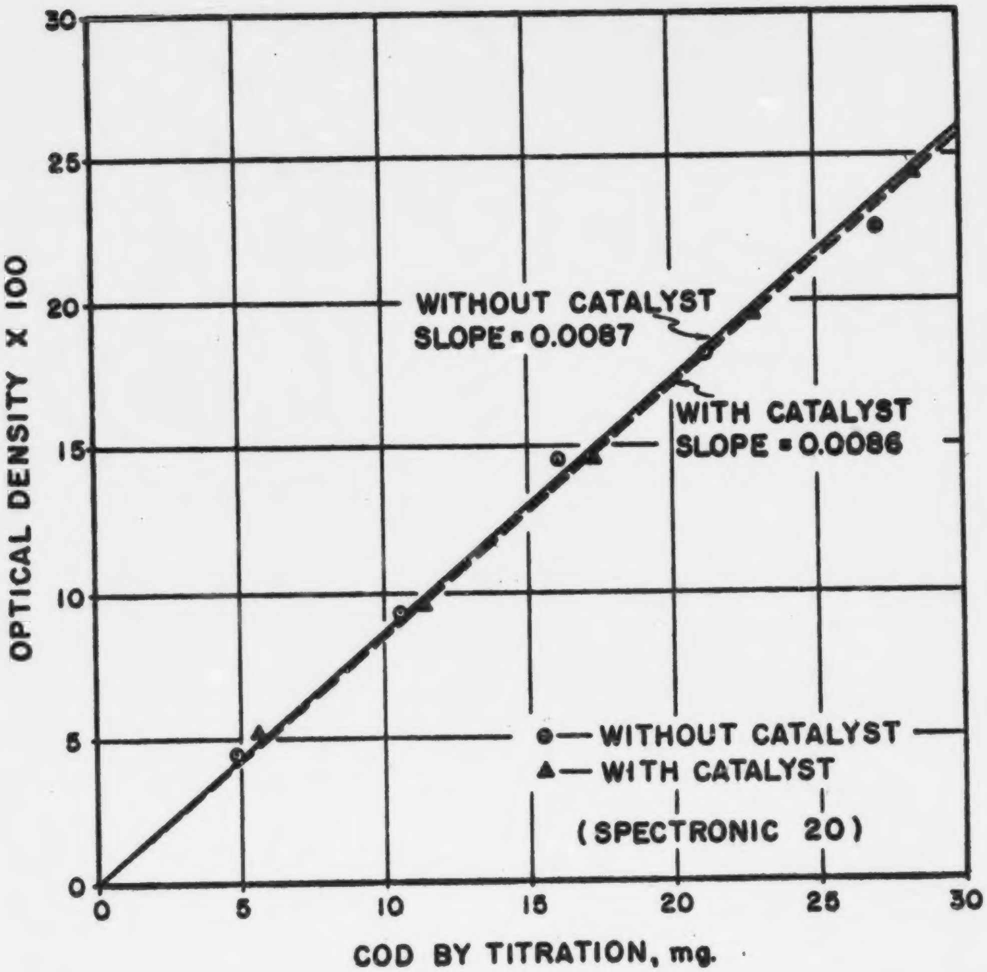

Fig. 3—Relationship Between Optical Density and COD, Refinery Waste.

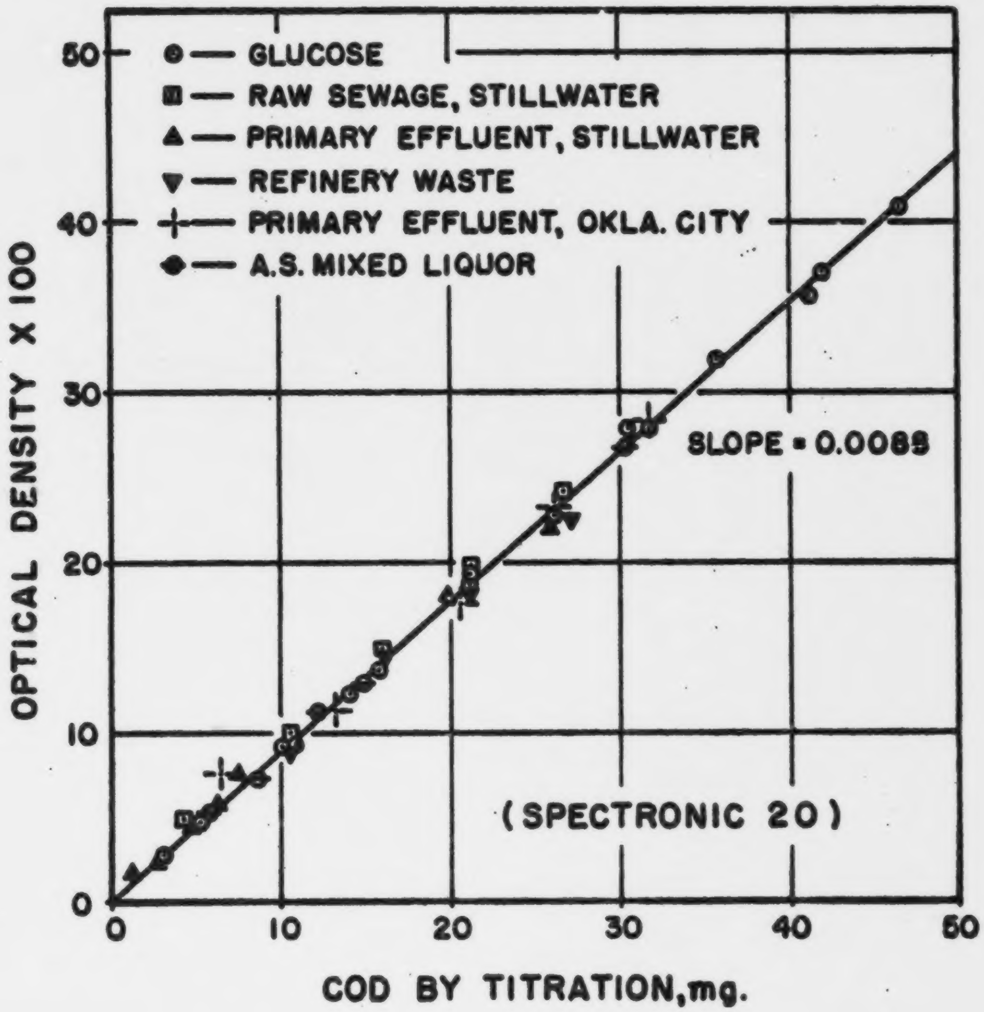

Fig. 4—Relationship Between Optical Density and COD, Catalyst not Added.

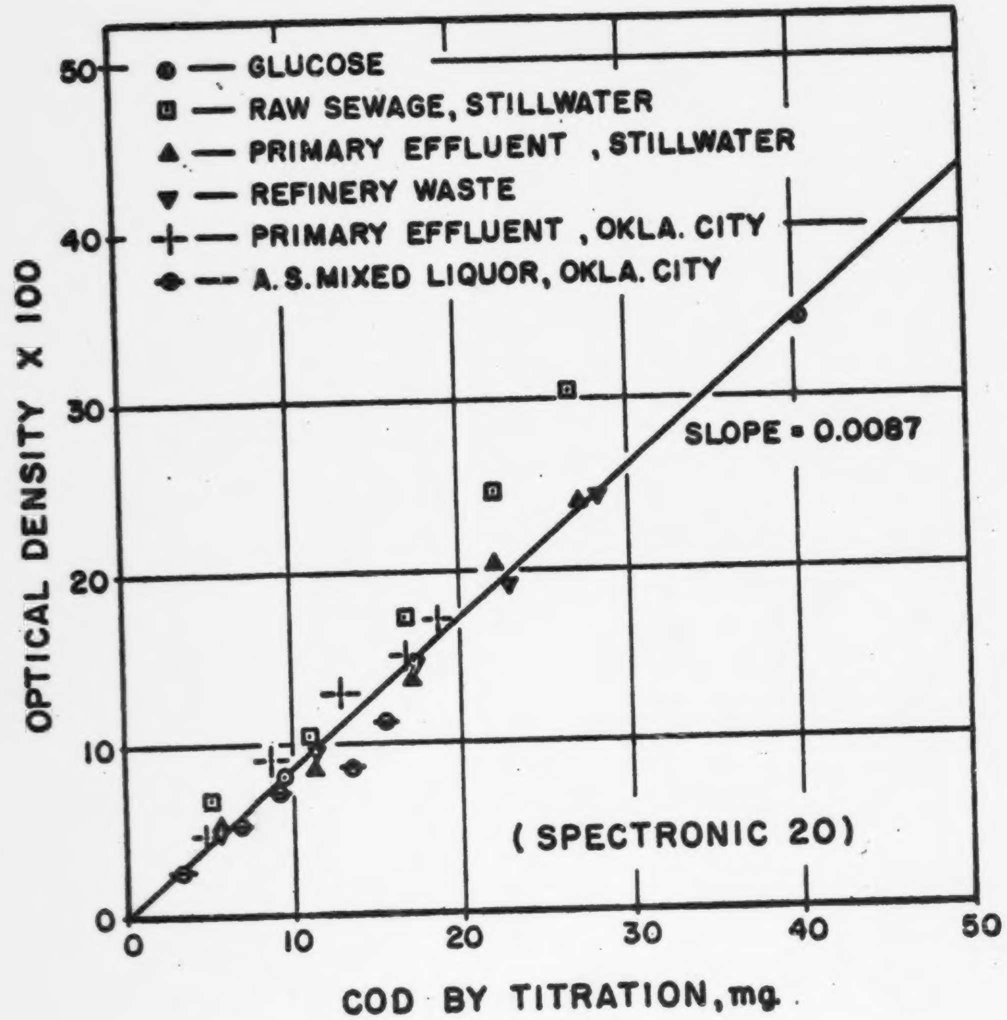

Fig. 5—Relationship Between Optical Density and COD, Catalyst Added.

density and COD remained the same with and without the catalyst. The reason for the greater dispersion of the points for the other wastes was observed to be the formation of a precipitate when the catalyst was added. The turbidity in some samples after refluxing interfered with measurement of optical density. Chloride concentration was not measured for any of the samples, but the refinery waste was known to contain only a trace amount whereas all the other waste samples, with the exception of the glucose standard, would be expected to contain a fairly high concentration.

Samples of white water, digester liquor and total plant effluent from a hardboard mill were also used to compare the titration and colorimetric techniques. The data for these wastes are shown in Figure 6 for COD's refluxed without the silver sulfate catalyst. The line of best fit yields a slope of 0.00825, which compares favorably with that found for the glucose standard (0.00834).

Figure 7 shows the correlation between the titration and colorimetric methods for these wastes when refluxed using the silver sulfate catalyst. For these wastes the addition of the catalyst yielded an even closer grouping of the data to the line of best fit and a slope of 0.00834. For these wastes which contained only slight amounts of chloride, no trace of turbidity was observed.

When $AgSO_4$ was added, the dispersion of the data for samples from municipal treatment plants appeared to be due to the formation of a precipitate which logically could arise because of the presence of chlorides; therefore it was desirable to initiate separate studies for the purpose of assessing the effect of chlorides on the colorimetric procedure.

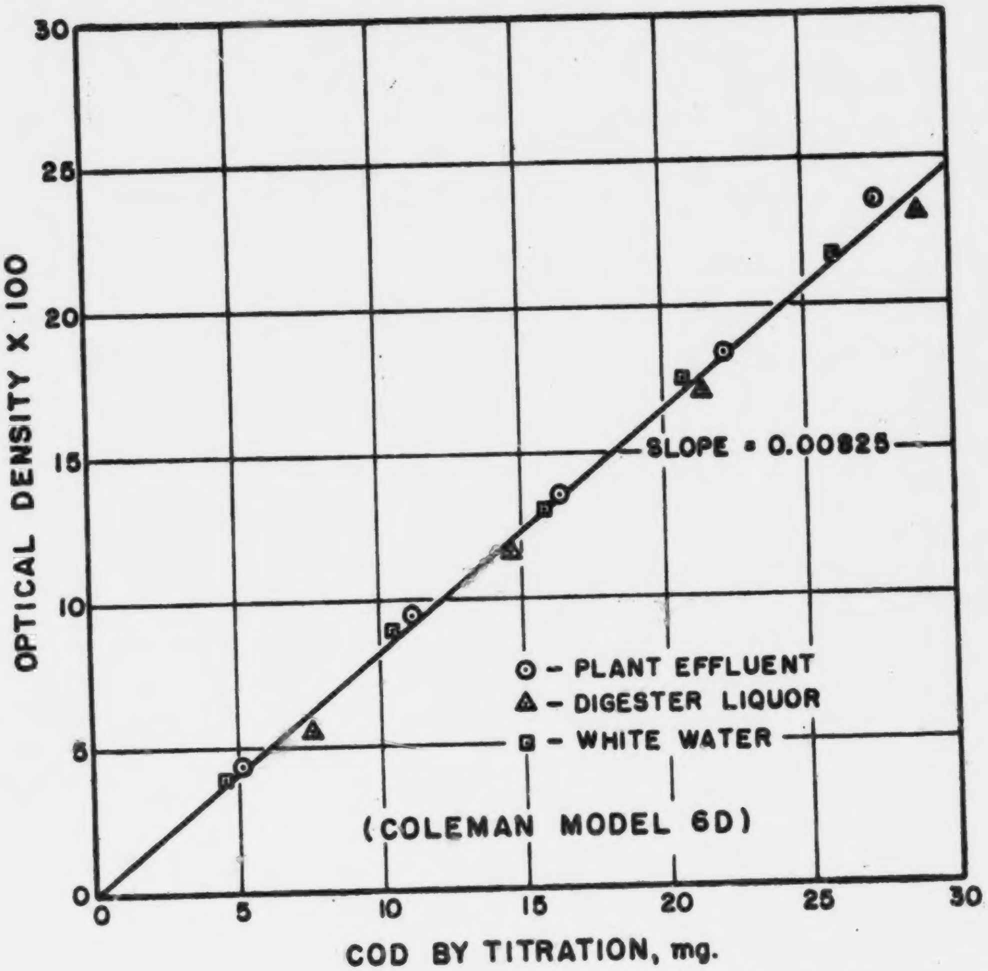

Fig. 6–Relationship Between Optical Density and COD, Catalyst Not Added.

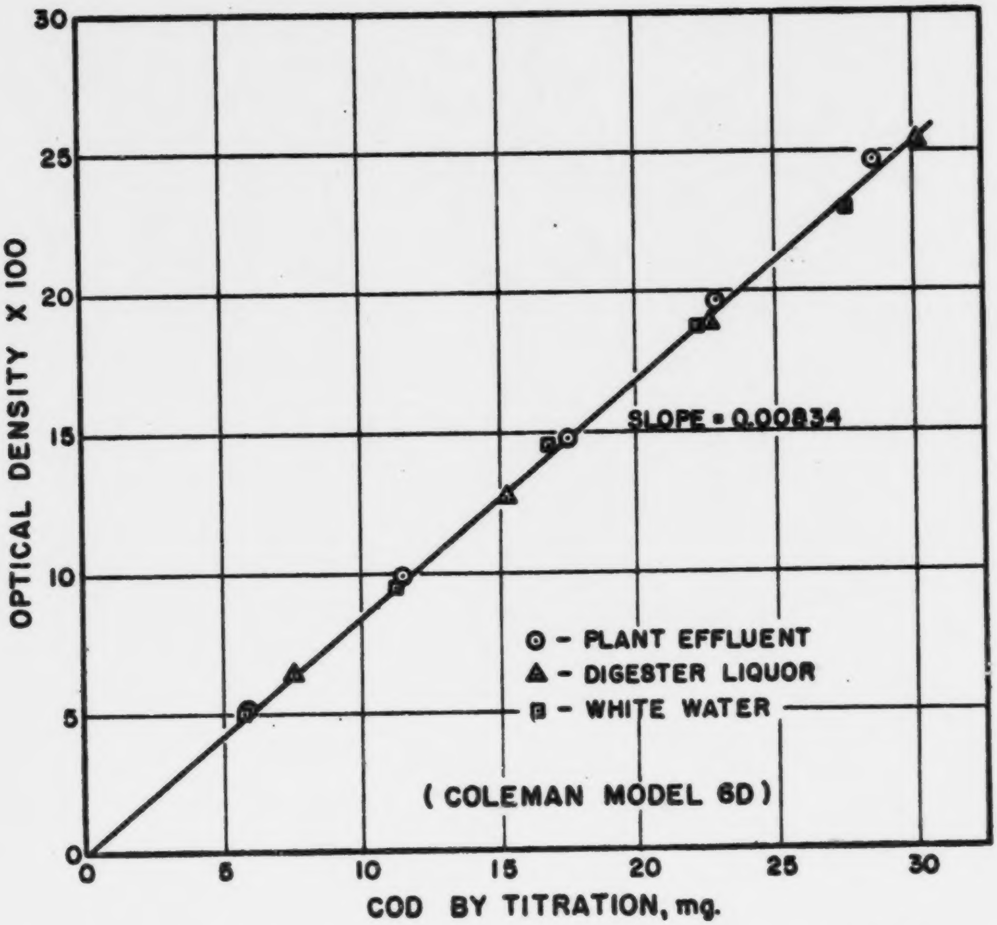

Fig. 7 - Relationship Between Optical Density and COD, Catalyst Added.

Accordingly, a study was made using glucose at 600 mg/l with varying concentrations of chlorides added as NaCl. Three conditions were examined: catalyst added at the beginning of the reflux period; no catalyst added; and catalyst added after the two-hour reflux period, with samples refluxed for an additional half hour. The latter procedure has been used by Bertram, it al.(10) The results of this study are shown in Table 1 for chloride concentrations varying from 50 to 400 mg/l; the second column shows the theoretical COD of the combined glucose and chlorides (COD_{th}). The COD's determined by titration and by optical density using the glucose calibration curve are also given, as are the ratios of COD determined colorimetrically to COD by titration (COD_c/COD_t), the COD determined by titration to the theoretical COD (COD_t/COD_{th}), and the COD determined colorimetrically to the theoretical COD (COD_c/COD_{th}). Comparison of the three conditions studies shows the effect of turbidity caused by the precipitation of silver chloride. Turbidity was noted only when the catalyst was added at the beginning of the reflux period, and then only when the concentration of chloride reached 200 mg/l. The presence of chloride ion did not seriously affect the ratio COD_c/COD_t below the 400 mg/l chloride level. When the catalyst was not added, the samples were always clear and the ratios COD_c/COD_t are in general more constant and closer to unity than those observed when the catalyst was added. Adding the catalyst after the two-hour reflux period with an additional one-half hour reflux prevented the formation of any discernible turbidity, and the high ratio obtained at the 400 mg/l chloride level when silver sulfate was initially added was reduced to

TABLE 1

Effect of Chlorides on COD_t and COD_c. Conditions: A, using $AgSO_4$, B, without $AgSO_4$ and C, refluxed for 2 hours followed by addition of $AgSO_4$ and ½ hour additional reflux.

	Cl^- mg/ℓ	COD_{th} mg	COD_t mg	COD_c mg	$\frac{COD_c}{COD_t}$	$\frac{COD_t}{COD_{th}}$	$\frac{COD_c}{COD_{th}}$
A	50	32.6	30.9	31.5	1.02	0.948	0.966
	100	33.2	31.6	33.0	1.04	0.952	0.994
	200	34.3	31.8	32.8	1.03	0.927	0.956
	300	35.5	31.6	33.3	1.05	0.891	0.938
	400	36.6	31.8	39.2	1.23	0.869	1.071
B	50	32.6	31.4	32.0	1.02	0.963	0.982
	100	33.2	32.8	32.0	0.977	0.986	0.964
	200	34.3	33.6	33.0	0.982	0.980	0.962
	300	35.5	34.8	34.0	0.978	0.979	0.958
	400	36.6	36.0	34.5	0.958	0.984	0.943
C	50	32.6	31.4	32.5	1.04	0.963	0.997
	100	33.2	31.7	33.0	1.04	0.955	0.994
	200	34.3	33.4	34.0	1.02	0.974	0.991
	300	35.5	34.4	36.0	1.05	0.969	1.014
	400	36.6	35.8	37.0	1.03	0.978	1.011

COD_{th} = Theoretical COD = mg glucose $\left(\frac{192}{180}\right)$ + mg Cl^- (0.23)
COD_t = COD by titration
COD_c = COD by colorimetry using glucose standard curve
All calculations based on 50mℓ samples

an acceptable value. In cases where the sample is known to contain chlorides, the latter procedure offers a method which would allow use of the colorimetric method with the catalyst in the presence of high chloride concentrations.

Turbidity is also an important factor in performing COD analyses by titration since it can interfere with the determination of the end point. The reflux procedure used in case C is therefore useful for either method of COD determination when chlorides are present in perceptible quantity. In any case it should be recognized that the use of silver sulfate (at the beginning of the reflux period) is best considered as an adjunct rather than as a replacement for COD's run without the catalyst.(11)

Further examination of Table 1 shows that there is fairly good agreement between the titrated COD and the theoretical COD. The least favorable comparison was found for the case where silver sulfate was added at the start of the reflux period. It would appear that the catalyst interfered with, rather than enhanced, oxidation at all levels of chloride concentration. At the 300 and 400 mg/l concentrations the primary interference appeared to be in determining the endpoint.

The COD values for all wastes studies are given in Table 2. This table was compiled by calculating the COD obtained from duplicate samples of at least five dilutions for each waste. These figures, therefore, represent the average of at least ten COD determinations per waste for COD's run with and without the catalyst added at the beginning of the reflux period. Figures given in parentheses represent COD's run in the presence of the catalyst. The ratio of COD obtained

NO.	DESCRIPTION OF SAMPLE	CHEMICAL OXYGEN DEMAND mg/ℓ		$\frac{COD_c}{COD_t}$
		COLORIMETER[1]	TITRATION	
1	RAW SEWAGE, STILLWATER	555 (684)[2]	516 (540)	1.076 (1.267)
2	PRIMARY EFFLUENT, STILLWATER	488 (550)	511 (563)	0.955 (0.977)
3	REFINERY WASTE, TULSA	5210 (5530)	5256 (5715)	0.991 (0.968)
4	PRIMARY EFFLUENT, OKLA. CITY	693 (470)	662 (430)	1.047 (1.093)
5	MIXED LIQUOR, OKLA. CITY	2970 (2662)	2965 (3290)	1.002 (0.809)
6	HARDBOARD, PLANT EFFLUENT	1109 (1185)	1090 (1155)	1.014 (1.028)
7	HARDBOARD, DIGESTER LIQUOR $\left(\frac{1}{25}\text{ DIL.}\right)$	1318 (1489)	1458 (1520)	0.905 (0.979)
8	HARDBOARD, WHITE WATER $\left(\frac{1}{10}\text{ DIL.}\right)$	511 (568)	507 (562)	1.008 (1.010)

Table 2

COMPARISON OF COD VALUES OBTAINED BY COLORIMETER AND BY TITRATION

1. Values obtained using glucose calibration curve, figure 2.
2. Values in parentheses are results obtained using $AgSO_4$ added at the beginning of the reflux period.

by colorimetry to COD by titration is also shown for all experiments.

Without addition of $AgSO_4$ the ratio COD_c/COD_t varied from 1.076 to 0.905, i.e., from +7.6 to -9.5 percent, with an average ratio of 0.999 or -0.1 percent for all wastes examined. When using the catalyst (added at the beginning of the reflux period) the ratio COD_c/COD_t varied from 1.267 to 0.809, i.e., from +26.7 to -19.1 percent with an average ratio of 1.016 or +1.6 percent for all wastes examined.

SUMMARY AND CONCLUSIONS

A thorough study comparing results of COD determinations by the standard titrimetric procedure and by colorimetry has been made using a variety of waste waters. The results indicate that the two procedures yield comparable COD values. The correlation between these methods is very good when the turbidity of the refluxed sample is low. If addition of the catalyst at the beginning of the reflux period leads to precipitation of silver chloride, an alternate procedure wherein the sample is refluxed in the absence of $AgSO_4$ followed by refluxing in its presence has been shown to avoid the formation of a precipitate when high chloride concentrations are present.

We have purposefully avoided using a shortened reflux period or altering the normalities, etc., recommended in Standard Methods. Such modification may, after experimental validation, be applicable to many individual situations. Our sole purpose was to determine whether colorimetry could be used as a reliable replacement for the titration procedure.

The colorimetric test herein presented provides a rapid and quantitative procedure which yields results comparable to those obtained by standard titration technique. In general the colorimetric procedure requires less technical skill and this, together with its speed, may encourage more extensive laboratory control at treatment plants.

ACKNOWLEDGEMENT

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PAPER 2

STATISTICAL STUDY OF THE SPOT-PLATE TECHNIQUE FOR VIABLE-CELL COUNTS

By

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This paper was publichsed in Applied Microbiology, 11, 305 (1962).

STATISTICAL STUDY OF THE SPOT-PLATE TECHNIQUE FOR VIABLE-CELL COUNTS

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ABSTRACT

Gaudy, A. F. Jr., (Oklahoma State University, Stillwater), F. Abu-Niaaj, and E. T. Gaudy. Statistical study of the spot-plate technique for viable-cell counts. Appl. Microbiol. 11, 305-309, 1963. -- A statistical study of the spot-plate technique was made for the purpose of establishing an acceptable counting range. The organism used in these studies was Micrococcus lysodeikticus. Standard deviations and coefficients of variation were computed for counts ranging from 20 to 440. The lower and upper limits for acceptable counts, based on coefficients of variation of 10 and 5.8 respectively, were chosen as 100 and 300.

Recent investigations in our laboratory required the counting of viable cells in many experimental samples. Previous experience suggested the use of the surface or "spot-plating" technique. This method had been successfully used in studying the succession of predominance in systems containing up to four aerobic bacterial species (Gaudy, 1955). The method involves application of a known volume of bacterial suspension onto the surface of solidified agar. In the work cited above, the bacterial count was based upon a total sample volume of 0.08 ml applied in four 0.02-ml spots. Four spots can easily be placed on one-half of a standard petri dish. This allows plating of two counting units on one dish. This counting technique has also been successfully used in water pollution control research for estimating the survival of Salmonella typhosa in high-rate anaerobic sludge digestion (McKinney, Langley, and

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Tomlinson, 1958). Although the method has many operational advantages, the relatively small volume plated (0.08 ml as compared with 1.0 ml for pour plates) precludes its use in estimating viable count in samples of very low bacterial density.

A statistical study of the pour-plate and spot-plate techniques, employing the coefficient of variation (c.v.) as the major parameter for comparison, showed both methods to be equally reliable; however, the spot-plate method yielded slightly higher counts. Comparison of the counts, using the Student's t test, showed that the difference was significant at the 95% level, thereby indicating that it was due to inherent differences in the methods (Gaudy, 1955). Since the distinct morphological characteristics of the organisms used in the study provided ample assurance of the absence of contamination, it was reasoned that the method yielding the higher count would be more accurate. The more abundant air supply and absence of temperature shock when making spot plates were considered to be possible causes for the higher viable count. These studies served as a basis for concluding that the spot-plate method yielded equally reliable and slightly more accurate population estimates than the pour-plate method. Although these conclusions were not in total accord with those of Pomales-Lebrón and Fernández (1952), who felt that the methods were equivalent, the results of both studies may be taken as recommendations for greater use of the surface-plating technique.

In a recent study, Stapert, Sokolski, and Northam (1962) examined the effect of temperature upon bacterial counts obtained with pour plates and the membrane filtration technique. They concluded that the consistently higher count, which was obtained when using the filtration technique, was attributable mainly to the fact that the bacterial suspension was not subjected to the temperature of liquid agar. The use of the membrane filter technique for counting requires special equipment which may not be available in all laboratories. The spot-plate method, however, can be used in any laboratory equipped to use the pour-plate method and possesses the same advantages of surface growth and lack of temperature shock.

In the work previously cited (Gaudy, 1955), the upper and lower limits for counting were taken as 5 to 40 colonies per spot, corresponding to 20 to 160 per half plate counting unit. This range was not selected as an optimal one based upon direct experimental evidence. Its selection was based largely upon comparison with the limits of 30 to 300 colonies usually employed for pour plates and consideration of the plate area available for growth in both methods. Since the spot-plating method appeared to provide a useful research tool, it was felt that further studies were warranted. These were designed to establish the limits within which viable counts obtained with the spot-plate method would be considered acceptable in our laboratory. No limits were generally applicable to all cases, due to variations in colony size of the organisms used or to the exact requirements of any particular study as to counting precision. However, the data obtained in this study offer additional evidence of the statistical validity of spot-plate counts, and the methods which were employed in choosing limits for this particular study may be useful to others who wish to use the spot-plate technique.

MATERIALS AND METHODS

Test organism. Micrococcus lysodeikticus (Fleming, 1922) was chosen as the test organism because it forms rather small rounded colonies on nutrient agar and possesses easily distinguishable morphological characteristics which facilitate detection of contamination. (M. lysodeikticus Purdue strain was kindly furnished by E. A. Grula, Microbiology Department.) The organism was grown on nutrient agar slants (Difco) throughout the study and was transplanted on fresh agar each week.

Experimental protocol. Nutrient agar plates were poured and dried at 37 C. for 48 to 72 hr. The drying time is somewhat dependent upon the drying temperature and the volume of agar used, but should be sufficiently long to eliminate any surface moisture from the plates. The bottom of each petri dish was marked with a bisecting line, thus providing two plating areas per dish. Standard dishes (100 mm diameter) provided ample area to accommodate two counting units. A 0.1-ml pipette, graduated in 0.01-ml divisions, was used to place four spots of 0.02 ml each onto agar surface. The summation of colony counts from these four spots (0.08 ml) comprised the counting unit upon which the population estimate was based. If the plates were sufficiently dried before use, a 0.02-ml volume would spread to a spot with a diameter of 15 mm or less, and would be absorbed by the agar in 10 to 15 min. After the liquid had been absorbed, the plates were incubated at 37°C for 48 hr. and then counted with the aid of a Quebec colony counter.

Replicate plating for statistical analysis. The experimental design for these studies required replicate platings of the test organism at colony counts ranging from 5 to 110 colonies per spot. To approximate each range an optical density (OD)-viable count relation was determined for a suspension of the test organism. In setting up any particular experiment, a suspension was prepared, its OD was measured, and dilutions were prepared in accordance with the OD-viable count relationship previously determined. Replicate counting units were then spotted for each of the desired ranges thus estimated. All colony counts could then be used to calculate the viable population in the original suspension from which the dilutions were prepared. Four such studies, hereafter referred to as studies A, B, C, and D are reported. The number of replicate counting units (n) plated for each counting range varied from 22 to 10.

Statistical formulas. The statistical analysis employed was based primarily upon formulas given by Stearman (1955) and Fisher (1946). The variation within each set of replicate counts was expressed as the standard deviation (s) calculated according to the equation

$$s = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}} \qquad (1)$$

where $\bar{x}$ is the arithmetic mean of the individual counts (x), and n is the number of plates counted.

Direct comparison of the variation in the counts obtained in different counting ranges was then made by dividing each standard deviation by its mean ($\bar{x}$) and expressing this ratio as a per cent, i.e., the coefficient of variation.

$$\text{c.v.} = \frac{100(s)}{\bar{x}} \qquad (2)$$

The numbers of bacteria in a series of samples taken from a single suspension should approximate a Poisson distribution if the method used for estimating cell numbers introduced no error other than the random sampling error. The applicability of the Poisson distribution is based on an infinitely large number of possible events and a small (< 0.10) value of p which is the probability of success (Croxton, 1959). These requirements are fulfilled by the removal of small samples from a large population of cells, since the total number of bacteria (n) is large and the probability of including any one cell in a sample is equal to the ratio of sample volume to total volume. Additional criteria are based upon the requirement that the bacteria be randomly distributed. This condition is obtained if (i) the cells do not repel one another or if there is sufficient space between cells so that any repelling effect is negligible, (ii) the relative volume of cells is small with respect to the volume of liquid, and (iii) there is no clumping of cells (Stearman, 1955).

The chi-square test was used to determine whether the variation in observed counts was significantly greater than would be predicted on the basis of a Poisson distribution using equation 3 (Fisher, 1946).

$$X^2 = \frac{\sum (x - \bar{x})^2}{\bar{x}} \qquad (3)$$

Since the number of parallel samples obtained was not large enough to provide an adequate test of agreement with the frequencies predicted by a Poissonian distribution of X^2 values, a total X^2 was calculated using the method described by Fisher (1946). According to this approximation, the difference between the quantities $\sqrt{2X^2}$ and $\sqrt{2n} - 1$ should be no greater than 1.645 if the over-all level of variability is no greater than expected at the 95% confidence level.

The theoretical standard deviation of the mean in a series of sample means is given by the formula

$$\sigma_{\bar{x}} = \frac{\sigma}{\sqrt{n}} \tag{4}$$

This quantity is also often called the standard error of the mean. Using a range of 2 $\sigma_{\bar{x}}$, the per cent precision for any sample mean at the 95% confidence level may be computed as the range divided by the mean:

$$\text{Precision (\%)} = \frac{2\sigma_{\bar{x}}(100)}{\bar{x}} \quad \text{or} \quad \frac{2s}{\bar{x}\sqrt{n}}(100) \tag{5}$$

TABLE I

STATISTICAL ANALYSIS OF SPOT-PLATE COUNTS*

Study		Viable count							Per cent precision
		n	$\bar{x}$	per ml	s	c.v.	χ^2	P	
A	1	22	11.7	$1.4x10^8$	2.6	22.2	12.2	0.95	9.5
	2	22	29.2	$1.4x10^8$	5.1	17.5	18.7	0.6	7.5
	3	22	49.5	$1.4x10^8$	6.6	13.3	18.5	0.6	5.7
	4	22	77.7	$1.4x10^8$	7.9	10.2	16.9	0.7	4.3
	5	22	108.9	$1.4x10^8$	12.1	11.1	28.2	0.1	4.8
	6	22	127.7	$1.3x10^8$	13.9	10.9	31.8	0.05	4.6
	7	22	164.2	$1.2x10^8$	14.7	8.9	27.6	0.2	3.8
B	8	22	224.9	$5.2x10^7$	14.1	6.3	18.6	0.6	2.7
	9	20	336.1	$4.9x10^7$	23.3	6.9	30.7	0.05	3.1
C	10	12	24.1	$4.5x10^7$	4.8	19.9	10.5	0.5	11.5
	11	12	126.8	$4.8x10^7$	7.3	5.8	4.6	0.95	3.3
	12	12	229.6	$4.8x10^7$	13.6	5.9	8.9	0.7	3.4
	13	12	277.2	$4.7x10^7$	15.3	5.5	9.3	0.6	3.2
	14	12	322.3	$4.6x10^7$	18.8	5.8	12.0	0.3	3.4
	15	12	358.5	$4.5x10^7$	19.9	5.6	12.1	0.3	3.2
	16	12	390.6	$4.4x10^7$	20.6	5.3	11.9	0.4	3.1
	17	12	430.8	$4.2x10^7$	28.2	6.5	20.3	0.05	3.9
	18	12	442.4	$3.9x10^7$	21.1	4.8	11.1	0.4	2.8
C	19	10	5.2	$1.6x10^7$	2.0	38.4	6.9	0.7	24.4
	20	10	41.6	$1.7x10^7$	8.8	21.2	16.8	0.05	13.4
	21	10	92.7	$1.6x10^7$	11.0	11.9	11.7	0.25	7.5
	22	10	134.2	$1.5x10^7$	15.0	11.2	15.1	0.1	7.1

*Symbols: n + number of plates counted; $\bar{x}$ = arithmetic mean of individual counts; s = standard deviation; c.v. = coefficient of variation; P = value from table of X^2 (Croxton, 1959).

TABLE II

TOTAL χ^2 FOR COUNTS OBTAINED USING THE SPOT-PLATE TECHNIQUE

No. plates in set	No. of sets	Σ (n)	Total χ^2
22	8	168	172.5
20	1	19	30.7
12	9	99	100.8
10	4	36	50.5
Total	-	322	354.5

The expected per cent precision for random samples distributed according to the Poisson distribution may be calculated for any values of n and μ according to the same formula, which may be reduced to equation 6 by substitution of the $\sqrt{u}$ for σ and μ for $\bar{x}$

$$\text{Expected per cent precision} = \frac{2\,\sigma_{\bar{x}}(100)}{\mu}$$

Since $\sigma_{\bar{x}} = \sigma / \sqrt{n}$ and $\sigma = \sqrt{\mu}$

$$\text{Precision (\%)} = \frac{2\,\sqrt{\mu}(100)}{\mu\,\sqrt{n}} = \frac{200}{\sqrt{\mu n}} \qquad (6)$$

Equation 6 was used to construct a family of curves depicting the variation in per cent precision with average plate count at selected values of n.

RESULTS AND DISCUSSION

The arithmetic mean and standard deviation for each set of plate counts in the four studies are shown in Table I. The X^2 value for each set of counts, calculated according to equation 3, is also shown. Values of P corresponding to each X^2 (for n - 1 degrees of freedom) were taken from the X^2 table as given by Croxton (1959). If the limits of significant deviation are taken as P = 0.05 and P = 0.95 (Fisher, 1946) then the counts obtained by the spot-plate method may be concluded to be no more variable than expected on the basis of random sampling. However, six sets of parallel plates (A-1, A-6, C-11, C-17, and D-20) lie very close to the limits of expected variability. A-1 and and C-11 were less variable than would be expected in approximately 95% of cases, and the other four sets of counts were more variable than expected by chance in approximately 95% of cases.

As a further test of agreement with the expected Poisson distribution of counts, the total X^2 for all counts was calculated as shown in Table II. From these data, the value of $\sqrt{2X^2} - \sqrt{2n - 1}$ was computed as 1.3. As previously stated, a value less than 1.645 indicates that the general level of variability of counts does not exceed that expected at the 95% confidence level. Therefore, it seemed reasonable to conclude that the spot-plate technique offered a reliable method for making viable-cell counts, and the data obtained was used in selecting upper and lower counting limits.

In Figure 1 coefficients of variation are plotted against the average colony count for studies A, B, C, and D. The coefficient of variation is quite high for the lower counting ranges but decreases rapidly with increasing count, reaching a fairly constant value in the higher counting ranges. The viable count for each study, calculated from the mean for each counting range, is shown in Table I. There was a slight decrease in viable count as the number of colonies per spot increased.

In selecting acceptable upper and lower counting limits, it would seem unwise to use a range over which there was a wide change in variability; hence it was desirable to make use of a portion of the coefficient of variation-count curve in which the coefficient of variation was not changing rapidly. The selection of an upper limit cannot be made solely by a consideration of this curve since the coefficient of variation remains constant as the number of colonies per counting unit increases. However, there is a tendency for the viable count to decrease as the number of colonies per spot increases (Table I). This effect is usually attributed to crowding, and may be brought about by nutritional shortages which prevent all

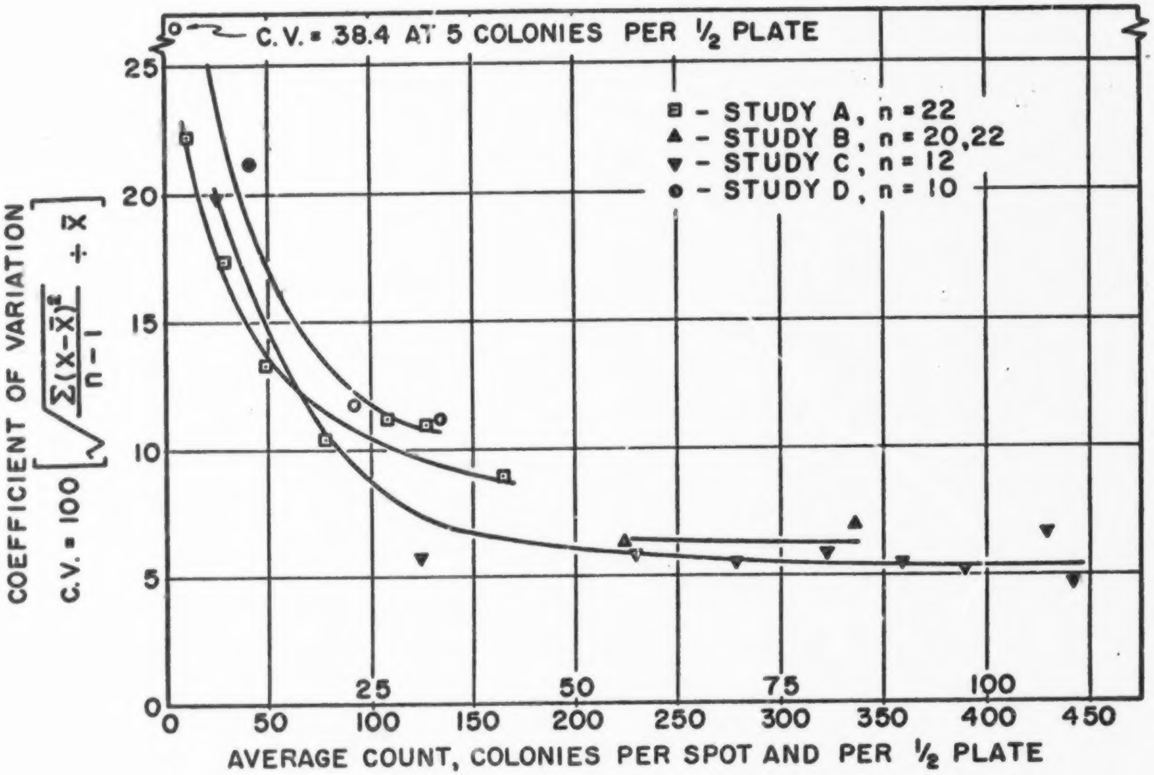

Fig. 1–Relation between coefficient of variation and colony count for "Spot Plate" surface counting technique.

the organisms from developing into visible colonies. It may also be due to inability of the analyst to make an accurate count in the higher ranges. In these studies, difficulty was experienced in counting plates with over 75 colonies per spot.

In an effort to delineate individual capability of counting in the higher ranges, a panel study was made in which four analysts counted the same set of plates covering a range of approximately 200 to 450 colonies per half plate (Table III). Analysts were ranked in order of experience in using the spot-plate technique; analyst 1 was the most experienced, and analyst 4 had no previous experience in counting surface plates. All four expressed difficulty in counting, beginning with the third counting range; however, it is seen that the average counts obtained by each investigator are in close agreement, regardless of the counting range. Based upon the information gained in this study and the results shown in Figure 1, a tentative upper limit was set at 75 colonies per spot, or 300 colonies per counting unit.

It is interesting to note that an upper limit of 300 colonies is usually cited for pour plates, although the area used is much greater for this method than for the spot-plate technique. Based upon the present study, it seems difficult to envision the upper limit for pour plates as being necessitated by a nutrient deficiency due to the crowding of many organisms in a small area. However, it does seem highly probable that an analyst would experience more difficulty in counting 300 colonies spaced over the entire plate area and depth, rather than in four surface spots of approximately 1.3 cm diameter.

TABLE III

AVERAGE REPLICATE COUNTS OBTAINED BY FOUR ANALYSTS COUNTING THE SAME PLATES

Analyst	Avg count*	Coef of variation	Viable cells per ml
			X 10^{-7}
First counting range			
1	215	5.5	5.9
2	220	8.3	6.1
3	215	8.2	5.9
4	218	13.7	6.0
Second counting range			
1	299	6.0	6.2
2	296	7.0	6.1
3	290	6.7	6.0
4	298	7.8	6.1
Third counting range			
1	358	8.4	5.9
2	363	10.8	6.0
3	359	9.0	5.9
4	363	10.3	6.0
Fourth counting range			
1	421	6.5	5.8
2	459	10.7	6.3
3	434	10.8	6.0
4	442	8.1	6.1

*Each average based upon 12 counting units.

If the lower limit of 30 colonies per counting unit usually considered acceptable for pour plates were applied to the spot-plate data shown in Figure 1, it would place the lower limit on a rapidly falling portion of the curve and indicate a spread in coefficient of variation from 20 to 6 within the accepted counting range. For most work, such a wide variation is undesirable and, to use the flat portion of the curve, a lower limit of 80 to 100 would seem advantageous.

The per cent precision computed by equation 5, using s computed by equation 1, is shown in Table I. The per cent precision for the observed mean counts of Studies A, C, and D are plotted in Figure 2, which also shows the theoretical curves for per cent precision for various values of μ at selected values of n computed from equation 6. In general, the per cent precisions computed from the experimental data lie close to the respective theoretical curves, with the exception of those for sets of counts for which the X^2 test indicated excessive variability. The theoretical curves are useful as a guide in experimental design or as an interpretive aid in assessing the per cent precision of count data.

For many experiments in our laboratory, we employ duplicate plating (n = 2) and accept a lower limit of 100 colonies. On this basis, the per cent precision varies from 14.2% at the lower counting limit to 8.2% at the upper limit (300 colonies). The theoretical coefficients of variation for the lower and upper limits are then 10 and 5.8, respectively. Referring to Figure 1, it is seen that coefficient of variation computed from the experimental data does lie close to 10 at the lower limit and 5.8 at the upper counting limit.

In using these limits, it is desirable to plate fivefold as well as tenfold dilutions. It is important to note that the organism used in these studies was ideal because it formed small, well-rounded, easily discernible colonies. It might not be possible to use an upper limit of 300 for species with less ideal surface-colony characteristics. For Escherichia coli, which grows fairly rapidly with somewhat spreading colonies, we employ counting limits of 100 to 300 colonies, but use a shorter incubation

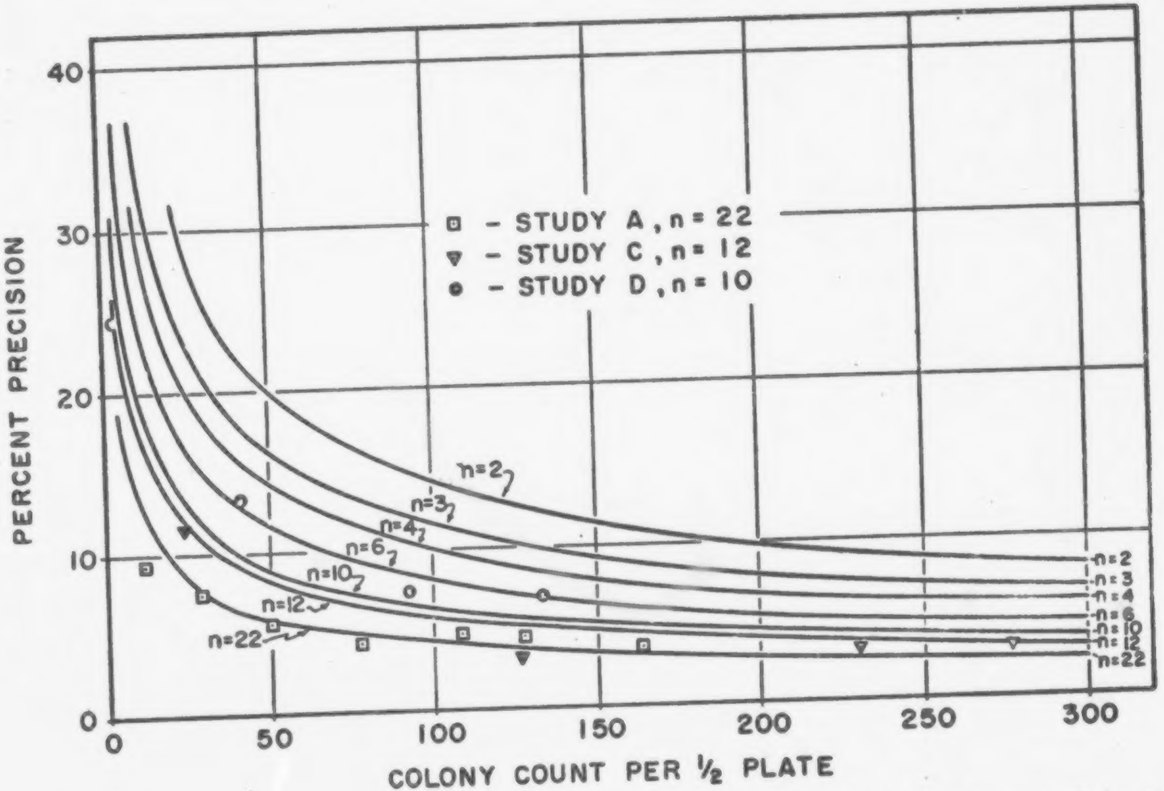

Fig. 2 - Theoretical and observed percent precision for various values of n.

period than that herein reported.

The results and analysis presented are not intended to set forth unequivocal lower counting limits. These must be set in accordance with the precision required for a particular experiment and the purposes for which the data are to be used.

ACKNOWLEDGEMENT

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PAPER 3

USE OF CHEMICAL OXYGEN DEMAND VALUES OF BACTERIAL CELLS IN WASTE-WATER PURIFICATION

By

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USE OF CHEMICAL OXYGEN DEMAND VALUES OF BACTERIAL CELLS IN WASTE-WATER PURIFICATION

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ABSTRACT

Gaudy, A. F. Jr., (Oklahoma State University, Stillwater), M. N. Bhatla, and E. T. Gaudy. Use of chemical oxygen demand values of bacterial cells in waste-water purification. Appl. Microbiol. 12:254-260. 1964. - Four methods for determining substrate recoveries in studies concerned with the partition of substrate between sludge synthesis and respiration were investigated. An energy balance comparing chemical oxygen demand (COD) removed with the summation of oxygen uptake and the COD of the cells produced yielded average recoveries closer to 100% than any of the other three methods tested. The standard COD test was shown to yield highly reproducible values when used to determine the COD of activated sludge. Although the protein and carbohydrate content of the cells varied with cell age, a concomitant variation in cell COD was not noted.

The partition of exogenous substrate between synthesis and respiration is an important aspect of process selection and design in the biological treatment of waste waters. Prediction of the amount of excess sludge produced in the treatment process is necessary for the design of sludge-handling facilities; a knowledge of the proportion of substrate oxidized is important in determining the minimal air requirement for the process.

In some cases, the partition of substrate has been estimated simply by measuring oxygen utilization and chemical oxygen demand (COD) removal; the amount of COD not accounted for as oxygen uptake is assumed to be channelled into sludge synthesis. Such an estimate assumes (i) that there is a simple partition of substrate between respiration and synthesis, (ii) that the techniques employed are sufficiently accurate to measure such partition, and (iii) that no gross errors have been made in analysis or in calculation. None of these assumptions is sufficiently unreasonable to invalidate the use of such approaches in pilot-plant studies. However, in research concerned primarily with delineation of the basic relationships between respiration and synthesis during waste-water treatment, some means of checking the above assumptions is necessary. This may be done through calculation of materials or an energy balance, either of which requires independent measurements of substrate removal, oxidation of substrate, and conversion of substrate to cellular material.

Either a direct carbon balance or use of radioactive tracers would provide accurate data for computing substrate recovery. However, neither method is entirely applicable to the determination of substrate partition in waste-water studies. Carbon determinations cannot be converted directly to sludge mass or even to oxygen requirements without making assumptions as to the carbon content of the sludge mass and the ratio of CO_2 production to oxygen utilization. In addition, the carbon balance requires specialized equipment not often found in water pollution laboratories, and it is extremely time-consuming. Radioactive tracer techniques would be applicable in studies of synthetic wastes of known composition, but could not be applied to whole wastes.

Excluding carbon analysis and radioactive tracer techniques, there would appear to be four methods for estimating substrate recovery which utilize analytical procedures commonly employed in water pollution control research. All four methods necessarily involve measurement of the three parameters listed above, and differ primarily in the techniques employed for making these measurements or for converting the material measured to common units. The removal of substrate is commonly measured as the decrease in COD of the waste water. There are several advantages in using the COD measurement rather than the biochemical oxygen demand (BOD) or specific substrate tests. The COD is a nonspecific test which can be used for wastes of either known or unknown composition. It is preferable even for measurement of known substrates in synthetic wastes since, with few exceptions, it detects the presence of intermediates in the system and therefore approaches an accurate measurement of all substrate not completely oxidized or incorporated into cell material more nearly than is possible with assays for specific carbon sources. The excretion by the cells of partially oxidized intermediates would not affect the calculation of substrate utilization, since the COD these compounds exert would be reduced in proportion to the amount of biological oxidation (oxygen uptake) required to produce them. The BOD measurement cannot be used for calculation of substrate recovery balances because the substrate, in the BOD test, is also partitioned between respiration and synthesis.

Oxidation of substrate is commonly measured as oxygen uptake in a Warburg respirometer. Several methods are available for the measurement of the amount of substrate utilized for production of cell material. The variations of these techniques which may be combined to provide four methods of obtaining a substrate balance are described below. Each method was used to calculate the substrate balances herein presented.

Materials balance, weight calculation. Gaudy and Engelbrecht (1960) presented data and a materials balance based upon conversion of all measurements to weight of substrate. With synthetic wastes containing single known carbon sources, both COD and oxygen uptake measurements could be converted to equivalent weights of substrate.

A direct conversion of substrate mass to cell mass was assumed, and substrate utilized for cell synthesis was therefore measured as increase in dry weight of cells. This type of balance could not be used for wastes of unknown composition.

Materials balance, carbon calculation. If it is assumed that the carbon content of the sludge mass remains constant throughout the substrate removal period, a materials balance may be based upon an average carbon content of cell mass. The average carbon content of bacterial cells is usually taken as 50% (Luria, 1960). The empirical formula for activated sludge, $C_5H_7NO_2$, developed by Hoover and Porges (1952) yields a carbon content of 53.1%. Other investigators have presented slightly different formulas (Symons and McKinney, 1958). Based upon their survey of the literature, Servize and Bogan (1963) selected an average carbon content for activated sludge of 51%. This figure is herein employed. The increase in sludge mass can be converted to an equivalent amount of substrate carbon, and a materials balance can then be computed by comparing the amount of substrate carbon removed during any time period with the amount of substrate carbon channelled into synthesis and the amount of substrate carbon oxidized (assuming that CO_2 produced can be calculated from O_2 uptake). Since the COD value must be converted to substrate carbon, this method is applicable only to studies using known carbon sources.

This method is simply an approximation of an actual carbon balance and involves the same assumptions as to carbon content and CO_2 to O_2 ratio. However, since the balance calculations are secondary to calculations of oxygen requirement and sludge production, this method, in which the primary data are those which are of greatest interest, is preferable to a carbon analysis in which these data would be derived.

Both methods of calculating a materials balance are applicable only to wastes of known composition. The only generally applicable type of substrate balance would therefore appear to be one based on the partition of substrate energy.

Energy balance based on empirical formula for composition of activated sludge. In a formal discussion of the weight method for the materials balance described above, McKinney (1960) suggested assuming a common value for organic sludge solids COD for use in calculating an energy balance. Grady and Busch (1963) recently employed this approach for studies involving cell recovery techniques in a "total biochemical oxygen demand test." The COD value of the cells was calculated as 1.414 times the cell weight, based on the amount of oxygen theoretically required to completely oxidize material of the formula $C_5H_7NO_2$. The balance could then be calculated by comparing the reduction in COD of the waste at any time with the sum of the measured oxygen uptake and the COD of the cells computed from the measured weight.

It would seem preferable to use a more direct analytical approach rather than to employ a general empirical formula for sludge or to assume an average sludge solids COD.

Energy balance based on measurement of cell COD. In previous work (Gaudy and Engelbrecht, 1960), it had been noted that both nonproliferating and growing cells could remove substrate at approximately the same rate with approximately the same increase in solids. However, there was a considerable difference in the composition of the sludge produced. For example, with glucose, almost all of the increase in weight under nonproliferating conditions could be attributed to carbohydrate synthesis, whereas under growth conditions equal increases in weight were noted, but the increase was almost totally attributable to protein synthesis. Thus, if the sludge composition could be so drastically variable, so, too, might be the empirical formula for sludge. Also, as a result of variable chemical composition, the chemical oxygen demand of the cellular material might be highly variable. In addition, such factors as cell age might be expected to change cell composition, or the chemical nature of the substrate could affect cell composition (Herbert, 1961). All of these factors could tend to alter the elemental composition of the biological mass and its oxygen equivalent, thereby negating the validity of using an oxygen equivalent based upon a general empirical formula. However, if individual experimental values of cell COD could be employed, a rapid and general method of assessing substrate recovery under any operational conditions would ensue without the need to make a general assumption concerning the elemental composition or COD of the sludge mass.

In our recent studies, a direct analytical approach to providing a check on experimental techniques has been investigated and found to be highly satisfactory. In this method, COD removal is determined as a measure of substrate disappearance. Oxygen uptake in a Warburg respirometer is measured as a means of assessing substrate respired. In addition, the chemical oxygen demand of the sludge produced is measured by the standard COD technique. The COD removed in any given time period is then compared with the summation of oxygen uptake and the COD of the cells produced during this time period, thus providing an energy balance. Evaluation of this method and factors affecting the COD of the biological mass is the primary concern of this report.

MATERIALS AND METHODS

Methods of analysis. Biological solids were measured by the membrane-filter technique (Millipore Filter Corp., Bedford, Mass.; HA, 0.45 μ). The COD test (American Public Health Association, 1960), run on the membrane filtrate, was used for determination of total organic matter remaining in the medium. The COD test was also used for measuring the COD of the cell mass. The protein and carbohydrate contents of the cells were determined by the biuret and anthrone methods, respectively, as previously reported (Gaudy, 1962; Gaudy, Gaudy, and Komolrit, 1963a). Oxygen uptake was measured on a Warburg respirometer (Gilson Medical Electronics, Middleton, Wis.).

Experimental protocol. Heterogeneous populations were obtained from two batch-operated activated sludge units. One of these units was operated with a minimal salts medium with glucose as the sole source of carbon, and the other employed sorbitol. The units were started from an initial seed of settled effluent from the primary clarifier of the municipal sewage treatment plant, Stillwater, Oklahoma. The composition of the standard synthetic waste, as well as the daily feeding procedure, was previously described (Gaudy, et al. 1963a). For specific experiments, cells were harvested from these units, washed once in 0.05 M phosphate buffer (pH 7), and suspended in a small volume of buffer-salts solution of the same chemical composition as that in which the cells were grown. The cells were then suspended in a larger volume of buffer-salts medium such that the total volume of the system was

1.5 liters after addition of the carbon source. Either glucose or sorbitol was added to obtain the desired concentration in the experimental system. For experiments in which oxygen uptake data were obtained, samples were immediately placed on a Warburg respirometer. The equivalence of experimental conditions in the batch activated sludge tubes and the Warburg apparatus was previously determined and has been substantiated many times in our laboratory. At a temperature of 25 C. with an air-flow rate of 4,000 cc/min in the growth tubes and a Warburg shaker rate of 100 oscillations per min., rates of COD removal and solids production were the same in both the growth tubes and the Warburg flasks. Mixed liquor samples were withdrawn from the 1.5-liter experimental units at desired time intervals. For many experiments, desirable sampling times were located by following the progress of growth by use of optical density measurements at 540 mμ (model D-6 spectrophotometer, Coleman Instruments, Inc., Maywood, Ill.). Portions were removed and filtered (Millipore, HA) for determination of biological solids production, and for COD analyses on the cells. In some experiments portions were also removed and filtered to obtain cells for protein and carbohydrate analyses. In all experiments, substrate remaining was measured as COD of the filtrate. For some experiments, carbohydrate determinations on the filtrate were also made.

For experiments designed to determine the effect of cell age on the COD of the biological mass, three cell age designations were used: young, old, and intermediate-aged cells. These are essentially operational definitions, and were described elsewhere (Gaudy, Komolrit, and Bhatla, 1963b). Briefly, the designation "young" cells refers to cells taken for COD analyses near the end of the log phase in a culture started from a small inoculum of cells from the batch unit. "Old" cells are those taken from the batch activated sludge units, no earlier than 21 days after the units had come into solids balance. These cells exhibited the typical flocculating and settling characteristics of activated sludge. "Intermediate" age cells refer to those taken from the activated sludge unit after 3 days of batch operation. At this time, the system is not in solids balance, and the cells do not exist as flocculated masses.

RESULTS

Reproducibility of cell COD with the standard COD test. To determine whether any modification of the standard COD test would alter the apparent COD of the biological mass, two series of experiments were run. In the first, identical concentrations of cells were subjected to various reflux periods, ranging from the standard 2-hr. period to 24 hr. The results of a typical experiment with glucose-grown cells are shown in Table I. It is noted that the reagent blank used in computing these COD values was refluxed for the standard 2-hr. period. There was a slight increase of sample COD as reflux time was increased. However, comparison of the reagent blanks which had been refluxed for 2 and 24 hr. showed that an increase in the COD of the blank offsets the slight rise in COD of the sample. From studies such as these, it was concluded that the standard 2-hr reflux period would be satisfactory for determining the chemically oxidizable portion of the cell mass.

Table I

COD OF GLUCOSE-GROWN CELLS WITH VARIOUS REFLUX TIMES

Reflux time	COD*
hr	mg/liter
2	506
3	511
4	515
5	518
6	517
7	519
8	523
18	541
20	536
24	541

*Values based on a blank refluxed for 2 hr. Biological solids concentration in each sample was 354 mg per liter.

A second series of experiments was run with the standard 2-hr reflux time in which sludge concentration was varied from 200 to 1,000 mg per liter. For any specific sludge, the mg of COD per mg of dry solids remained constant. In all subsequent experimentation for which cell COD was determined, the biological solids concentration fell within these limits. Furthermore, as a continuing check on cell COD values, each time the cell COD was determined three different concentrations were refluxed. Therefore, all cell COD values herein reported represent the average of three separate determinations on three cell concentrations. It should be noted that cell COD values obtained in this way were identical for all three concentrations. From the results of the reflux time and cell concentration experiments, it is not possible to say that the cells were totally oxidized. However, these experiments provided assurance that the 2-hr reflux period was sufficient to oxidize that portion of the cells which could be oxidized by dichromate, and that the COD value obtained was highly reproducible.

Changes in system parameters during substrate removal by activated sludges. Figure 1 shows the typical response pattern during removal of glucose by a young heterogeneous population. The optimal sampling times were located by following growth in the system by means of optical density (not shown in the figure). It is seen that cell COD parallels solids production, and that the point of maximal cell production corresponds to the point of maximal COD removal. It is also seen that glucose, as measured by the anthrone test, was removed from the system more rapidly than was the total substrate COD. As with many other experiments in our laboratory employing young cells, this finding indicates that a fairly substantial portion of the original substrate can appear in the medium as metabolic intermediates, which can be oxidized by dichromate under the conditions of the standard COD test but are not responsive to the anthrone test. It is also seen that most of the rise in solids concentration is attributable to protein synthesis.

A total of 11 experiments like the one shown in Figure 1 were run to assess the effect of both cell age and the nature of the carbon source upon the COD and the protein and carbohydrate contents of the cells. The two carbon sources tested were glucose and sorbitol. For each substrate, cells of three different ages (young, intermediate, and old) were used as the initial cell inoculum; in each case cells had been grown on the same carbon source used for the experiment.

COD values for cells of different ages grown on glucose and sorbitol are shown in Tables II and III. For each experiment the

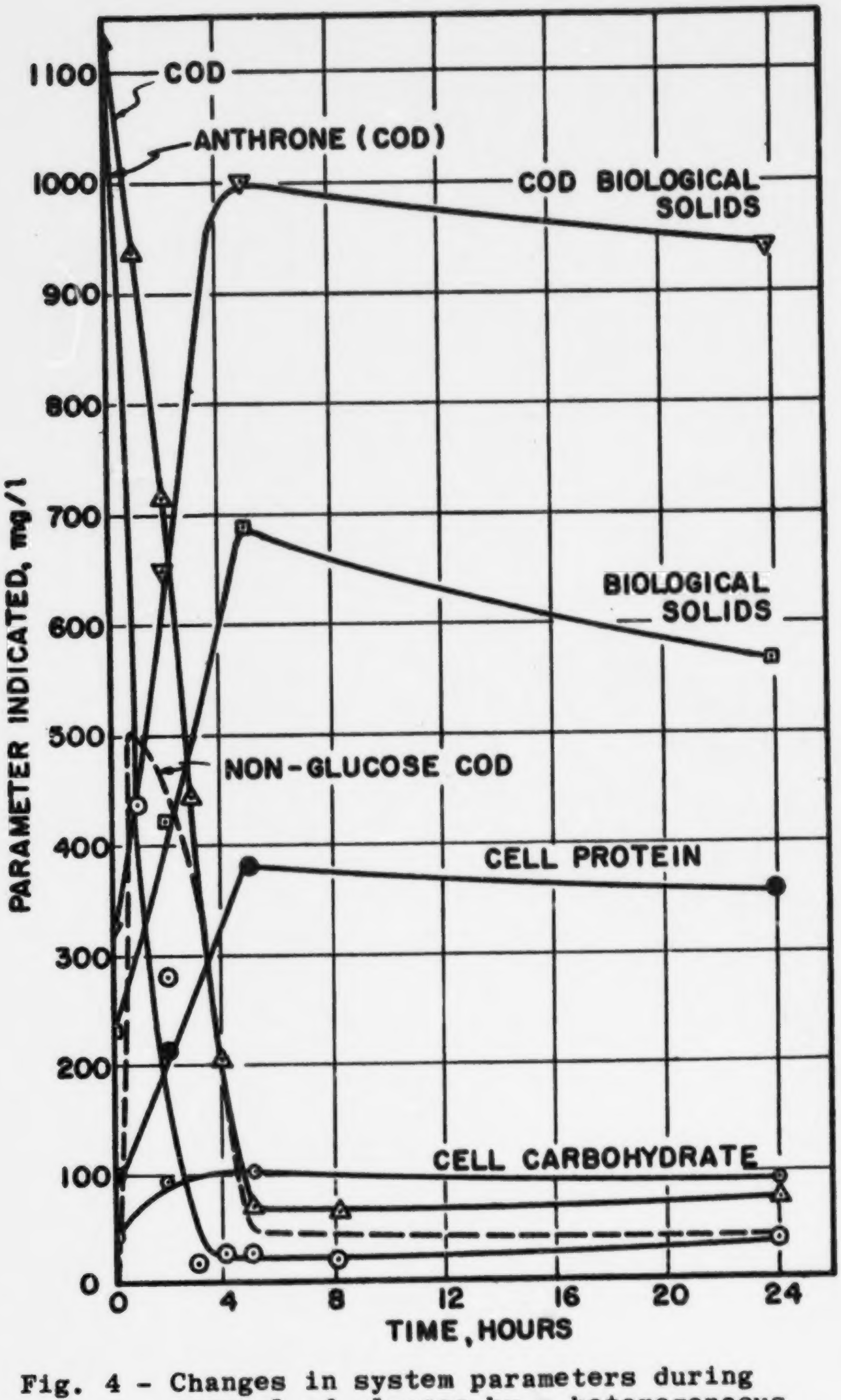

Fig. 4 - Changes in system parameters during removal of glucose by a heterogeneous biological population (young cells).

maximal and minimal COD values obtained during the run are given. The average value is the arithmetic mean of all COD determinations made during that particular experiment. COD determinations using three different amounts of cells are shown to demonstrate the reproducibility of the measurement.

A comparison of Tables II and III shows no consistent difference in the COD of cells grown on different carbon sources. Young cells grown on glucose exhibited higher COD values in two of three experiments than did young sorbitol-grown cells. However, in the third experiment using young glucose-grown cells and in those using intermediate or old cells, the substrate appears to have little effect on the COD of the cells.

Cell age also has no consistent effect upon cell COD. For glucose-grown cells, there appears to be a tendency toward higher COD values in younger cells, but the data are not sufficient to support a conclusion that COD varies with cell age. For sorbitol-grown cells the average COD is quite constant regardless of cell age.

The major conclusion to be drawn from these data is that use of an average value for cell COD may introduce considerable error in balance calculations. This is shown in the variation from the mean calculated for maximal and minimal values obtained for each run. With only these variations in the COD value for a single batch of cells during a cycle of substrate removal, the maximal range of variation from the mean was ±13.3% for glucose-grown cells (Table II) and -7.6 to +13.6% for sorbitol-grown cells (Table III). If an overall average COD value for all samples of cells grown on the same substrate is calculated and the varia-

TABLE II

RELATION BETWEEN COD OF CELLS AND DRY WEIGHT FOR VARIOUS CELL AGES DURING METABOLISM OF GLUCOSE BY GLUCOSE-GROWN CELLS*

Age Designation	Values during Growth Cycle	Dilution Factor			Avg Value	Variation from mean (%)
		1	2	4		
Young I	Maximum	1.65	1.69	-	1.67	-5.9 to +9.9
	Minimum	1.41	1.435	1.435	1.43	
	Average†				1.52	
Young II	Maximum	1.49	1.45	1.43	1.46	-11.8 to +7.3
	Minimum	1.19	1.2	1.21	1.20	
	Average				1.36	
Young III	Maximum	1.7	-	-	1.7	±13.3
	Minimum	1.35	1.25	-	1.3	
	Average				1.5	
Intermediate	Maximum	1.37	1.35	1.39	1.37	-5.4 to +6.2
	Minimum	1.22	1.22	1.22	1.22	
	Average				1.29	
Old	Maximum	1.23	1.25	1.27	1.25	-8.3 to +4.2
	Minimum	1.1	1.15	1.09	1.1	
	Average				1.2	

*Results are expressed as mg of COD per mg of solids (dry wt).

†Average values were computed from determinations of cell COD and dry weight for four to six samples taken at different times during glucose removal. Each sample was analyzed in triplicate at three different concentrations. Only values for samples with maximal and minimal COD are shown.

tion from the mean computed, the possible error involved in using an average COD value can be shown to be even greater. The overall average COD for glucose-grown cells is 1.37 mg of COD per mg of dry weight, with a variation from the mean of +24 to -20%. For sorbitol-grown cells, the overall average value is 1.31, with a range of +14.5 to -6.9%.

Since it had been shown in previous work that the protein and carbohydrate contents of cells could vary over wide ranges, it was of interest to determine whether the variation in either of these cell components could be correlated with variations in the COD of the cells. Therefore, during each experiment, pro-

TABLE III

RELATION BETWEEN COD OF CELLS AND DRY WEIGHT FOR VARIOUS CELL AGES DURING METABOLISM OF SORBITOL BY SORBITOL-GROWN CELLS*

Age Designation	Values during Growth Cycle	Dilution Factor			Avg Value	Variation from mean (%)
		1	2	4		
Young I	Maximum	1.29	1.32	1.32	1.31	±1.5
	Minimum	1.24	1.28	1.29	1.27	
	Average†				1.29	
Young II	Maximum	1.43	1.48	1.49	1.47	-7.5 to +10.5
	Minimum	1.2	1.26	1.22	1.23	
	Average				1.33	
Intermediate	Maximum	1.34	1.32	-	1.33	±3.1
	Minimum	1.27	1.22	-	1.25	
	Average				1.29	
Old I	Maximum	1.48	1.55	1.48	1.5	-7.6 to +13.6
	Minimum	1.22	1.22	-	1.22	
	Average				1.32	
Old II	Maximum	1.33	1.4	1.5	1.41	-2.2 to +5.2
	Minimum	1.28	1.35	-	1.31	
	Average				1.34	

*Results are expressed as mg of COD per mg of solids (dry wt).

†Average values were computed from determinations of cell COD and dry weight for four to six samples taken at different times during sorbitol removal. Each sample was analyzed in triplicate at three different concentrations. Only values for samples with maximal and minimal COD are shown.

tein and carbohydrate analyses were made for samples of cells taken at the beginning and end of the experiment, during rapid substrate removal and at the point of maximal solids production. These data showed that cell composition is greatly affected by operational conditions. However, there was no direct correlation between either protein or carbohydrate content and the COD of the cells. Protein varied from 12 to 63% of the cell dry weight, and the protein-COD ratios for cell samples varied from 0.10 to 0.42. Carbohydrate content of the cells varied from 7 to 34% of the cell dry weight, and carbohydrate-COD ratios varied from 0.05 to 0.35. Thus, although cell COD is variable, the effect upon cell COD of operational conditions which tend to foster high protein or carbohydrate contents cannot be predicted.

Comparison of substrate recoveries by use of various methods of computation. Table IV shows materials and energy balances calculated by each of the methods described above. Sample calculations for the 5-hr samples are shown below. In Table IV data obtained in an experiment using cells of intermediate age with glucose as carbon source were used for the balance calculations. For a second experiment for which balance calculations were made, cells were taken from an activated sludge unit which had been in continuous operation (24-hr batch feeding cycle) for more than 3 months. These cells were therefore designated as "old" cells. The carbon source for this unit and for the balance experiment was also glucose. Each of these experiments was carried out according to the experimental protocol described above, and samples were removed at the indicated times after addition of substrate.

From Table IV it is seen that recoveries calculated by method IV (an energy balance based on experimentally determined COD of the cells) were in general closer to 100% than recoveries calculated for the same experiment by any of the other three methods. For the experiment using old cells, recoveries were higher for all four methods than in the previous experiment. The numerical order of percentages recovered was the same in both cases; i.e., the methods may be arranged, for both experiments, in the same order according to decreasing percentage of substrate recovered: III > II > IV > I. Overall average per cent recoveries for the two experiments were: method I, 92.6; method II, 112.2; method III, 116.0; Method IV, 102.3.

TABLE IV

SUBSTRATE RECOVERY WITH VARIOUS METHODS OF COMPUTATION*

Time	COD rem mg/l	Cells mg/l	O_2 uptake mg/l	COD of cells mg/l	ΔCOD mg/l	ΔCells mg/l	ΔCOD cells mg/l	Calculated substrate recovery (per cent)†			
								I	II	III	IV
hr											
0.00	544	450		585							
0.5	450	485	13	626	94	35	41	53.5	65	66.5	57.5
1.5	292	573	45	730	252	123	145	70	84	87	75.5
2.0	231	629	64	810	313	179	225	81	98	102	93
2.5	143	715	84	922	401	265	337	91	110.5	114.5	105
3.0	72	755	101	985	472	305	400	90	109	113	106
3.5	26	764	116	960	518	314	375	87	105	108	95
4.0	20	774	129	970	524	324	385	90.5	109	112	98
4.5	20	777	138	1020	524	327	435	93	111	115	109
5.0	15	774	144	977	529	324	392	92.5	110.5	114	101

*Intermediate-age cells, acclimated to glucose

†(I) Materials balance, weight calculation. (II) Materials balance, carbon calculation. (III) Energy balance, based on empirical formula for composition of activated sludge. (IV) Energy balance based on measurement of cell COD.

Sample calculations for substrate recovery. Method I: Conversion factor for COD or O_2 to weight of substrate = 0.94, since 6 moles (192 g) of O_2 are required for complete oxidation of 1 mole (180 g) of glucose

$$\text{Recovery} = \frac{144(0.94) + 324}{529(0.94)} = 0.925$$

Method II: Assuming moles of CO_2 = moles of O_2 for glucose, then weight of CO_2 = weight of O_2 x 44/32, CO_2 carbon = 12/44 x weight of CO_2, cell carbon = weight of cells x 0.51, weight of substrate used = COD x 0.94, weight of substrate carbon used = weight of substrate used x 72/180.

$$\text{Recovery} = \frac{144(1.375)(0.273) + 324(0.51)}{529(0.94)(0.4)} = 1.105$$

Method III: Calculated COD of cells = 1.414 x weight of cells

$$\text{Recovery} = \frac{144 + 1.414(324)}{529} = 1.14$$

Method IV:

$$\text{Recovery} = \frac{144 + 392}{529} = 1.01$$

DISCUSSION

From the results herein presented it is concluded that an energy balance based on determinations of the COD of the culture filtrate, the COD of the cells produced, and the oxygen utilized by the cells can be recommended for use in waste treatment research. With two sets of data, balances were calculated by four different methods; the recommended method yielded better average recoveries than any of the others. The proposed balance technique would further appear to have a much sounder technical basis than the other methods, since it does not require the use of an empirical formula or average carbon content for activated sludge nor does it require that the chemical components of the waste be known. Thus, the recommended procedure is more widely applicable, since it may be employed with equal facility in studies on whole wastes or on synthetic wastes with known composition. If the composition of the waste is known, Method I may be preferable in some cases cince it yields adequate recovery data, and the analyses required are much less time-consuming than for Method IV.

In using cell COD to calculate an energy balance, it is not recommended that an average value for cell COD be employed to convert sludge weight to an equivalent COD. Whereas in some experiments the COD of the cells remained quite constant throughout the experimental period, in other experiments the COD of the cells varied over a broad range. In the latter cases a considerable error would have been introduced by use of an average COD value even though the average might have been experimentally determined for that particular sludge. Use of a general overall average COD value for cells might introduce even greater error. Our data indicate the possibility that both cell age and the particular substrate used may affect the COD of the cells, although not enough data are available to allow definite conclusions to be made in this respect.

The most valid objection to the use of the proposed method of determining substrate balances lies in the difficulty of demonstrating complete oxidation of cell material by the COD method. It is not possible to state that 100% oxidation of cellular material was achieved; however, the standard COD test does apparently oxidize all the cell material which acid dichromate is capable of oxidizing, since increasing the reflux time to as long as 24 hr. resulted in no further oxidation of the cells. COD values obtained with replicate samples of different amounts of cells were also highly reproducible.

Further support for the use of the COD test for activated sludges is provided by the recent report of Goldstein and Lokatz (1963) that the COD value of activated sludge provides a fairly accurate measure of its heat of combustion. According to their measurements, a value of 10 g per liter of COD corresponds to a heat of combustion of 500 BTU per gallon. Although this correlation does not provide absolute assurance that all organic matter in the cells is totally oxidized, it does provide evidence that a constant proportion of the organic matter in activated sludge is oxidized in the standard COD test. The fact that in our studies recoveries close to 100% were obtained with this measurement indicates that the proportion oxidized must be relatively high. Therefore, we believe that the standard COD test can be usefully employed in estimating the adequacy of data obtained in substrate partition experiments.

ACKNOWLEDGEMENT

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PAPER 4

STUDIES ON THE OCCURRENCE OF THE PLATEAU IN BOD EXERTION. I. HETEROGENEOUS AND PURE CULTURE SEEDS WITH VARIOUS CONCENTRATIONS OF GLUCOSE AS CARBON SOURCE

By

A. F. Gaudy, Jr., M. N. Bhatla, and F. Abu-Niaaj

Papers 4, 5, and 7 were originally prepared for separate journal publication. However, portions of Paper 4 and Paper 5 were combined as Paper 6 for presentation at the Purdue Industrial Waste Conference and Paper 7 was presented at the Oklahoma Industrial Waste Conference. Portions of all three papers (4, 5, and 7) were then combined as Paper 8 for publication in the W. P. C. F. Journal. Papers 4 and 5 are included here because they contain results not published in the summary papers.

STUDIES ON THE OCCURRENCE OF THE PLATEAU IN BOD EXERTION. I. HETEROGENEOUS AND PURE CULTURE SEEDS WITH VARIOUS CONCENTRATIONS OF GLUCOSE AS CARBON SOURCE

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Since its inception during the latter part of the nineteenth century, the BOD test has been the subject of much controversy and continuing research. It is used as a parameter in research, and it is also the subject of research. It is used as a "control" parameter at treatment plants. It is also a major basis for regulatory action. It is an integral part of dissolved oxygen sag curve analysis, and it forms a major basis for design of treatment units, yet, as stated by Hoover, Jasewicz and Porges, "No one appears to consider it adequately understood or well adapted to his work."(1) It is indeed the paradox these authors and many others have claimed it to be. Perhaps the fact that it has been called upon to serve such universal duty in a field which must deal with the full gamut of environmental variables helps explain in some measure why it cannot adequately serve all the purposes to which it has been put.

It would appear that until fairly recent times the major research effort involved proving that the kinetics of carbonaceous BOD expression were first order-decreasing rate, while during the past 25 or 30 years research efforts have been centered around

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Portions of this paper were presented at the 18th Industrial Waste Conference, Purdue University, 1963.

showing that the first order decreasing rate concept does not suffice to depict BOD kinetics. An examination of the literature shows the major kinetic concepts which have been applied to BOD exertion. These are depicted in Fig. 1. The concept first held and most prevalently used is the first order, continually decreasing rate type of kinetics which prevailed in the studies made by Theriault.(2) It has been stated many times that there is no fundamental reason to expect such kinetics. This is, however, a convenient way to express the course of BOD exertion, since only two constants, L_a and k, adequately describe the curve. Thus, in an engineering sense, it is highly desirable. From a theoretical standpoint, this type of kinetic curve might obtain in a system in which a limiting metabolite controlled the course of reaction. For example, biological systems devoid of an extracellular source of nitrogen (nonproliferating cell suspensions) have been observed to yield this type of oxygen uptake curve. Endogenous suspensions also exhibit this type of kinetics although both these systems more often follow zero order kinetics in the very early hours of observation.

Oxygen uptake curves best described as "autocatalytic" have also been observed.(3) The term "lag" has been applied to describe the early portion in which the curve is concave upward. It is recognized that this is not necessarily a lag in bacterial activity; however, in order to use a first order decreasing rate expression it must be treated as one. When the increasing rate portion is of short duration, the lag equation of Thomas would appear to provide a satisfactory treatment of the kinetics.(4) It is emphasized, however, that this increasing portion of the curve may often be shown to be first order, increasing rate. With

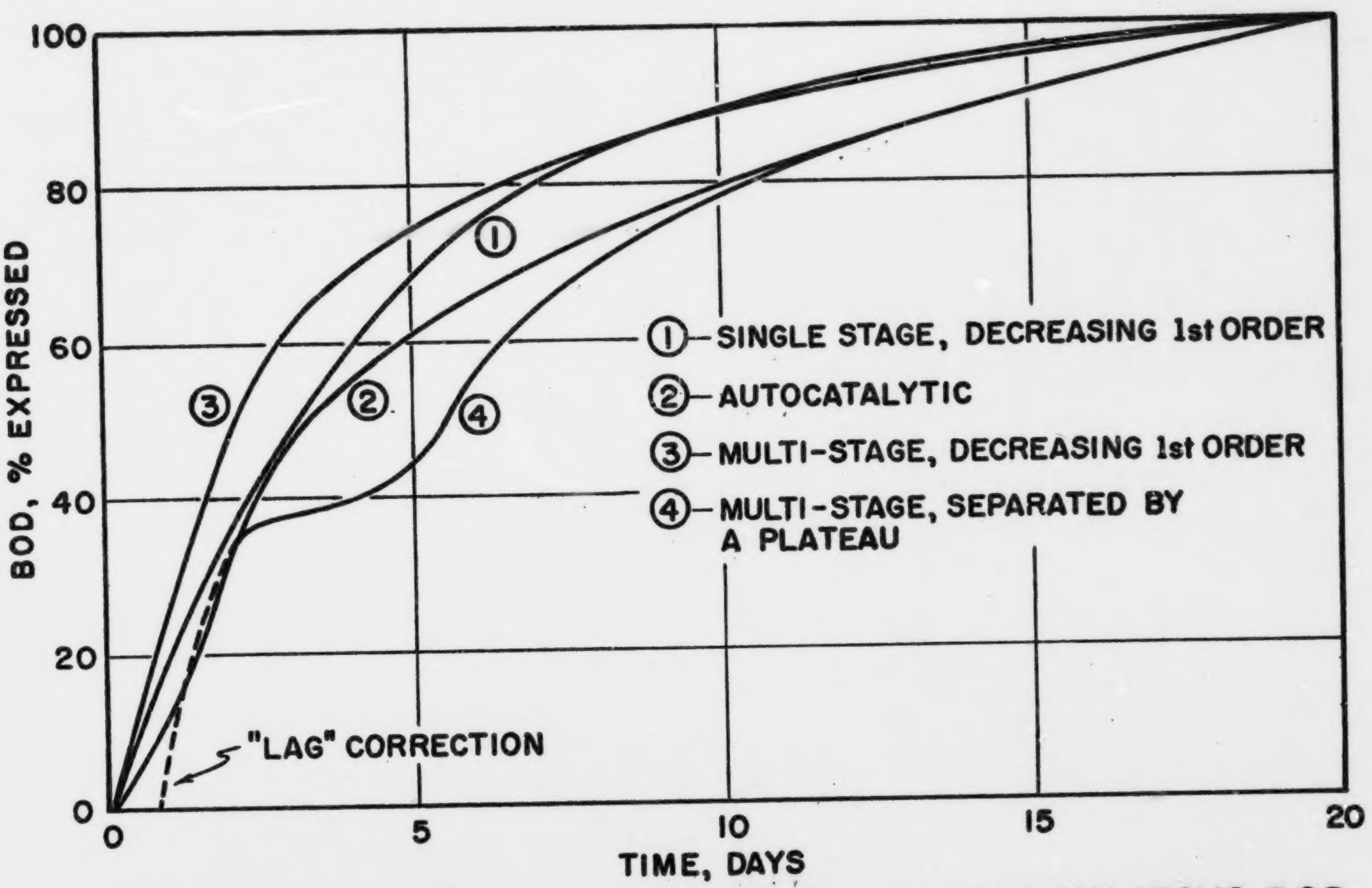

Fig. 1 - VARIOUS KINETIC EXPRESSION OF CARBONACEOUS BOD

an acclimated seed, this type of kinetics would be expected if the system was started using a low initial inoculum. The upsweeping portion would then correspond to a log growth phase and not a lag phase.

A third type of oxygen uptake curve for BOD exertion has been observed which can best be described as multiphase, first order decreasing rate wherein a rapid phase is followed by a slower one. (1)(5) In addition there may be more than two first order portions. This type of kinetics might be expected with somewhat high initial seed inocula in which the carbon source is not sufficient to support much new growth (as viable population increase), thereby preventing an autocatalytic curve. The separation of oxygen uptake into two phases has been explained as "rapid assimilation of substrates into carbohydrate storage products followed by a slower endogenous utilization of these intercellular products."(1)

As early as 1926 Greenfelder and Elder noted a second stage and suggested that it could be due to the death of organisms which were living at the time of sampling, and which, after dying, would become part of the carbonaceous oxygen demand.(6)

Recently Busch has observed another type of carbonaceous curve in which a rapid phase is followed by a second stage, but the two stages of oxygen uptake are separated by a plateau.(7) After the plateau, oxygen utilization in excess of endogenous respiration is attributed to the activity of the protozoa and other predators which feed on the bacteria. Except for the involvement of predators, the theory as stated by Busch is similar to that of Hoover, et al.(1) The difference lies in the plateau or pause in oxygen uptake before initiation of the second stage. Busch has suggested that "as the ratio of predators to substrate-consuming population increases, the separation of the two stages begins to blur."(7) He observed a reduced oxygen uptake after treatment of the seed to reduce the protozoan population. Wilson and Harrison have objected to Busch's conclusion that the rise in the second stage represented the joint effects of endogenous respiration and predator activity. (8) To test Busch's hypothesis they performed an experiment using a pure bacterial culture and phthalic acid substrate. In their experiment, oxygen uptake was measured on two systems containing different amounts of initial cell inoculum. They felt from their results that it was "apparent that the magnitude of the second stage of oxygen uptake depends solely on the number of cells present."(8)

McWhorter and Heukelekian have also investigated the occurrence of the plateau.(9) They varied substrate concentration (100, 500, 1000, 2000 mg/l glucose) and used 0.5 ml settled sewage as seed. Potassium nitrate was used as the nitrogen source. They found that the lag between phases (or the plateau) was shorter for 500 mg/l glucose than for the 100 mg/l concentration. The lag did not appear in the 1000 and 2000 mg/l systems. For the two systems exhibiting a plateau, they observed that while the increase in substrate concentration increased oxygen uptake, the accumulated uptake at the plateau expressed on a relative basis (percent theoretical) was the same for both systems. They also investigated the effect of predators and concluded that the disappearance of the plateau with increasing substrate concentrations could not be solely attributed to changes in the predator-bacteria ratio, since O_2 uptake curves for sonicated and normal seeds did not diverge until 45% of the theoretical demand had been expressed. This was above the plateau value which both they and Busch had obtained.

While there is slight disagreement concerning the masking of the plateau due to the predator-bacteria ratio, and while it must be said that there is no explanation for the occurrence of the plateau other than to say, as have Wilson and Harrison, that it suggests a delayed onset of endogenous respiration, the fact does remain that the plateau has been demonstrated for specific substrates by several independent research groups. It would thus seem that this type of kinetics must, if of general occurrence, be accorded consideration among the prevalent forms for carbonaceous BOD curves.

Some attempts have been made to extrapolate mechanistic principles from results obtained in higher energy systems to low energy systems such as those obtaining in the BOD bottle and in the receiving stream. This extrapolation is based on the assumption that the mechanisms are identical but merely go on at a slower rate in low energy systems. In a gross sense this would seem to be plausible, but it would appear that the fine scale mechanism which affects kinetics may not be the same in high and in low energy systems, or in any event may not be observable in both, as evidenced by the results of McWhorter and Heukelekian, who observed the plateau in Warburg experiments at 100 and 500 mg/l of substrate but not at 1000 and 2000 mg/l.

Our present interest in the BOD test arises out of studies we are undertaking concerning the mechanism and the kinetics of substrate removal in the activated sludge process. In studying kinetics, we believe that concurrent studies on mechanism to explain the observed kinetics must be undertaken. Since it was desirable to use the BOD test in combination with other analyses as a measure of system performance, it was necessary to investigate the occurrence of the plateau because of its serious ramifications in interpreting results of the standard test. In order to gain some insight into mechanism(s) of causation for the plateau, changes in bacterial population density during the course of BOD exertion were observed.

The studies herein reported were undertaken primarily to determine the generality or constancy of the occurrence of the plateau under various seeding conditions and substrate concentrations. Results using heterogeneous and pure culture seeds acclimated to glucose are presented.

MATERIALS AND METHODS

Analytical Techniques

Throughout the investigation the BOD and dissolved oxygen reagents used were those prescribed in Standard Methods for Examination of Water and Wastewater.(10) The Alsterberg modification of the Winkler method was used.

Estimates of bacterial population were obtained by the spot plate surface counting technique in which four spots of 0.02 ml each are placed on the surface of agar containing a suitable growth medium. Nutrient agar (Difco) was used in the studies herein reported. Previous experience had indicated that the method was as reliable and slightly more accurate than the pour plate method for aerobic organisms.(11) The method has also been successfully employed in studies on the survival of Salmonella typhosa in high rate digesters.(12)

Source of Seeding Material

In these studies the seeding material for various experiments was obtained from four sources.

Seed Type A:

This source of seeding material consisted of primary tank effluent from the Stillwater, Oklahoma, municipal sewage treatment plant. Such seed was obtained approximately one-half hour before use in a particular experiment. During this time the effluent was allowed to stand under quiescent conditions, which provided for further settling of suspended materials. The topmost portion of the effluent was used to seed the dilution water.

Seed Type B:

Seed for some experiments was obtained from the effluent of a laboratory batch operated activated sludge unit. The system was fed once each twenty-four hours. Prior to feeding, one-third of the mixed liquor was wasted; this was followed by quiescent settling for one hour and wastage of one-half of the remaining tank contents as effluent. A portion of such effluent was used as seeding material in the BOD studies. The batch activated sludge was developed from the initial seeding material described as seed type A.

Seed Type C:

Young cells developed from an initial inoculum of seed type B were also used as seeding organisms. These cells were harvested by centrifugation after fourteen hours of growth on a shaker apparatus (approximately the end of the log growth phase). The cells were washed three times in 0.05M phosphate buffer and used as seeding material in BOD studies.

Seed Type D:

A prototrophic strain of Escherichia coli was grown ascepticially on the shaker apparatus, harvested and washed as described for seed type C. All experiments using seed type D were conducted under aseptic conditions.

Substrates

The inorganic nutrients used in all BOD experiments were those described in Standard Methods for the Examination of Water and Wastewater. The exogenous carbon source consisted of glucose in varying concentrations. All seed types with the exception of type A were thoroughly acclimated to glucose. Type B seed was grown on a synthetic waste composed of (concentrations in mg/l); glucose, 1000; $(NH_4)_2SO_4$, 500; $MgSO_4 \cdot 7H_2O$, 100; $FeCl_3 \cdot 6H_2O$, 0.5; $MnSO_4 \cdot H_2O$, 10; $CaCl_2$, pH 7.5, These components were dissolved in 0.01M potassium phosphate buffer, pH 7.0, containing 100 ml of tap water per liter. Types C and D were grown in a medium consisting of the same constituents as those listed above with the exception that the carbon source was increased five-fold, salts two-fold, and buffer three-fold. This was done to insure a large harvest of cells and to prolong the log growth phase. Previous experience had indicated that the limiting metabolite for such a synthetic waste was the carbon source.

Experimental Protocol

Standard dilution water devoid of seed and substrate was equilibrated for twenty-four hours at 20°C. and aeration was begun thirty minutes prior to preparing BOD's for incubation. For each different system to be studied in a particular experiment, replicate portions of dilution water were taken and equal volumes of seeding material of the desired concentration were added to all portions. After adding substrate to the appropriate flask, at the desired concentration, both seed and sample + seed flasks were vigorously stirred and BOD bottles immediately filled. Samples of all systems were taken for initial dissolved oxygen concentration. Samples were also withdrawn for estimating the initial viable population. All BOD bottles were incubated, under a water seal, at 20°C. Bottles were removed periodically for measurement of dissolved oxygen remaining and changes in viable population. Samples for measuring the bacterial population were obtained by carefully withdrawing 1 ml from the BOD bottles immediately before adding the dissolved oxygen reagents. Appropriate dilutions were made using sterile distilled water. These were plated and incubated for twenty-four hours before counting. A preliminary study had shown that dissolved oxygen values were unaffected by the extra manipulations required in withdrawing 1 ml prior to adding the Winkler reagents.

RESULTS

Seed Type A

Fig. 2 shows the course of BOD exertion and changes in viable population for systems containing 8 and 5 mg/l of glucose using municipal sewage seeds. Multiphase kinetics were exhibited in both experiments regardless of the initial concentration of viable cells. In both experiments there is definite evidence for the plateau in BOD exertion. The first or rapid phase of oxygen uptake corresponds to a log phase of growth. The plateau occurs in the region of maximum viable count, and the last stage of oxygen uptake corresponds to a sharp decrease in viable popula-

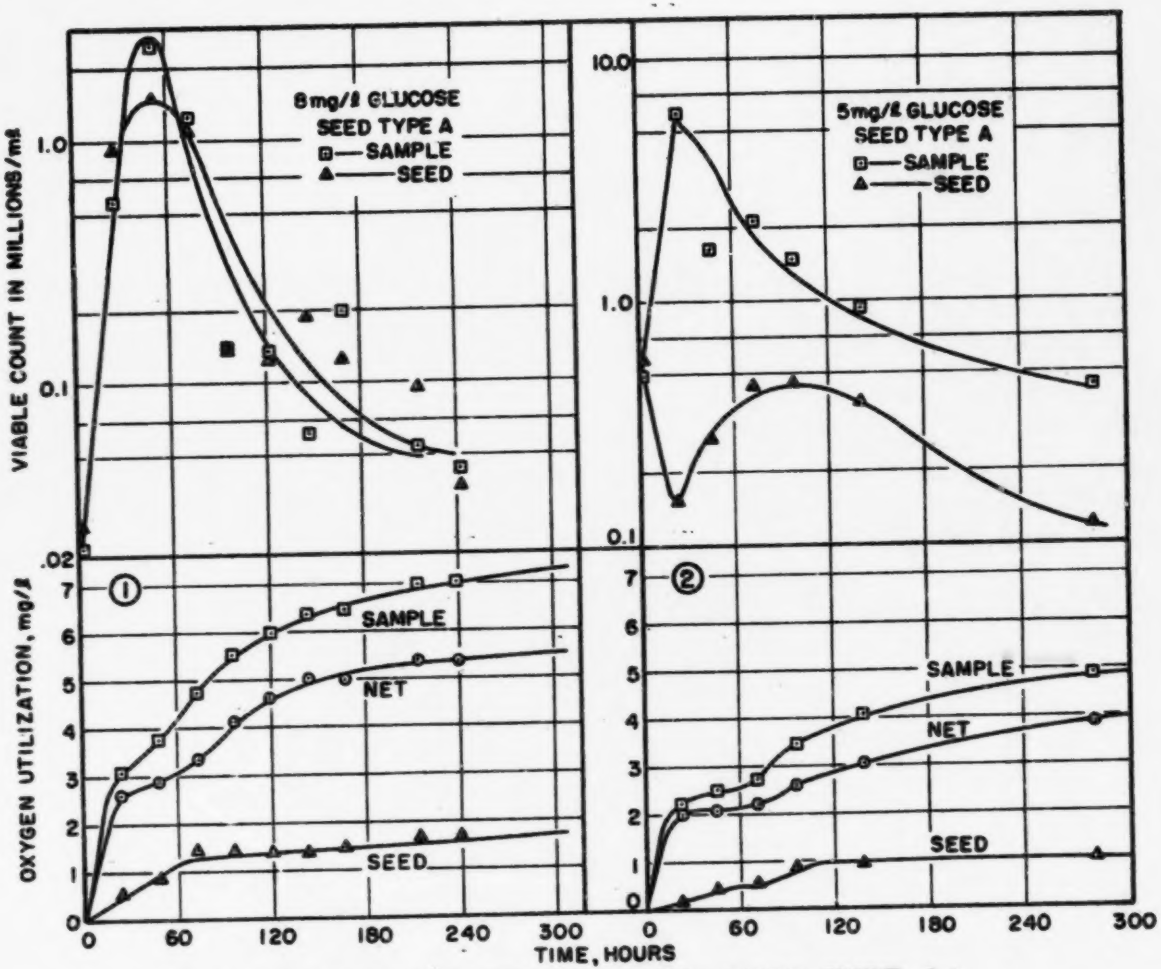

Fig. 2-BOD AND VIABLE BACTERIA COUNT, SEED TYPE A

tion. The behavior of the seed system is of particular interest. With a low initial inoculum ($2x10^4$) the viable count in the seed experiences a considerable log phase (approximately six doublings of the population), whereas in the other experiment there is an initial decrease in viable cells followed by a slight increase which in approximately seventy hours yielded a population density equal to that of the initial inoculum. The seed behavior may be attributed to the difference in viable population in the seed and to a variable strength of organic matter in the sewage from which the seed was obtained. Regardless of the variable nature of the seed, the plateau existed.

Seed Type B

Results for an experiment using seed obtained from the effluent of a laboratory activated sludge plant operating on glucose with sludge developed from an initial inoculum of municipal sewage is shown in Fig. 3. In this case there was very little oxygen used in the seed system. The population remained static during the first 120 hours, then gradually fell off. The system containing the sample shows the full gamut of BOD kinetics. There is a slight lag followed by an autocatalytic portion, both of which are reflected in the viable count data. The plateau is again in evidence, and corresponds to the range of maximum population density. The second phase of oxygen uptake again corresponds to a decrease in viable count. Oxygen uptake again becomes autocatalytic and finally approaches the typical first order decreasing rate curve which has been usually cited as describing the BOD reaction.

Seed Type C

Fig. 4 shows results using washed young heterogeneous seed de-

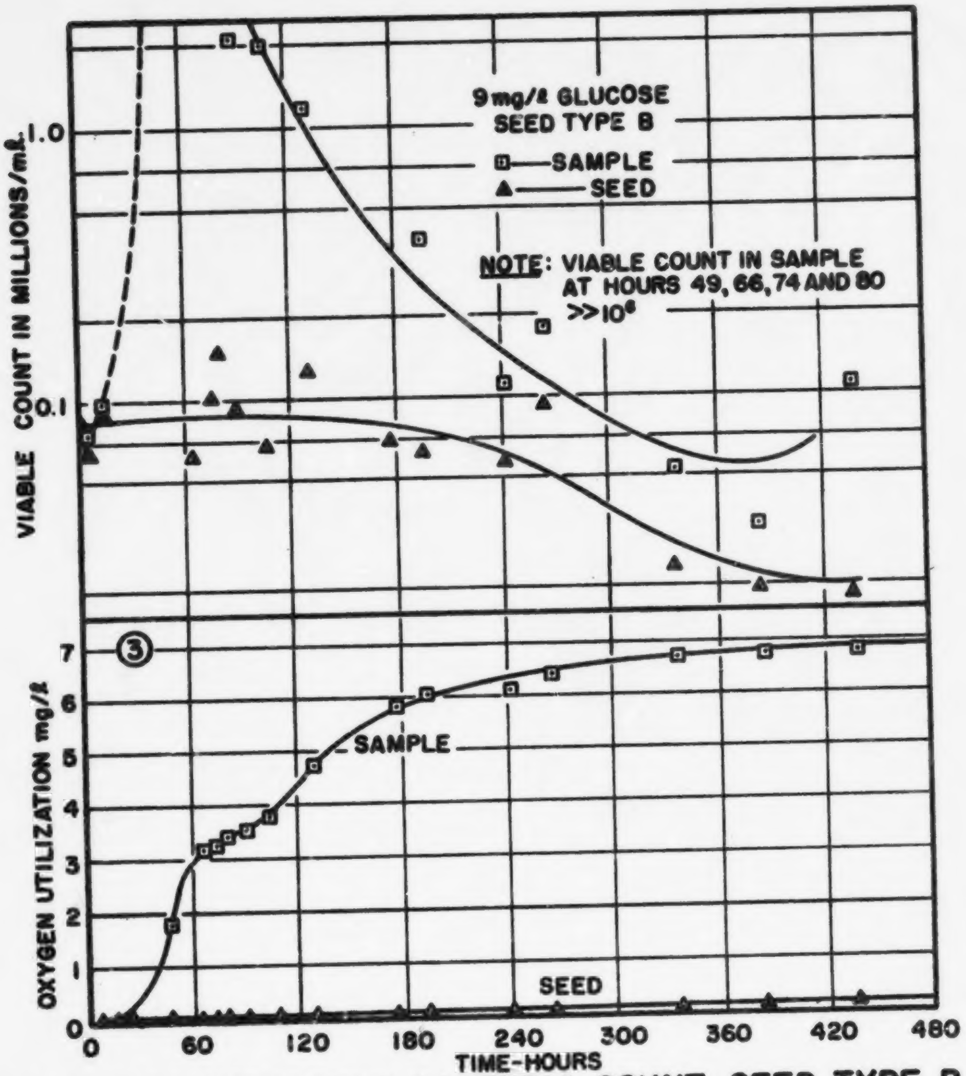

Fig. 3-BOD AND VIABLE BACTERIA COUNT, SEED TYPE B

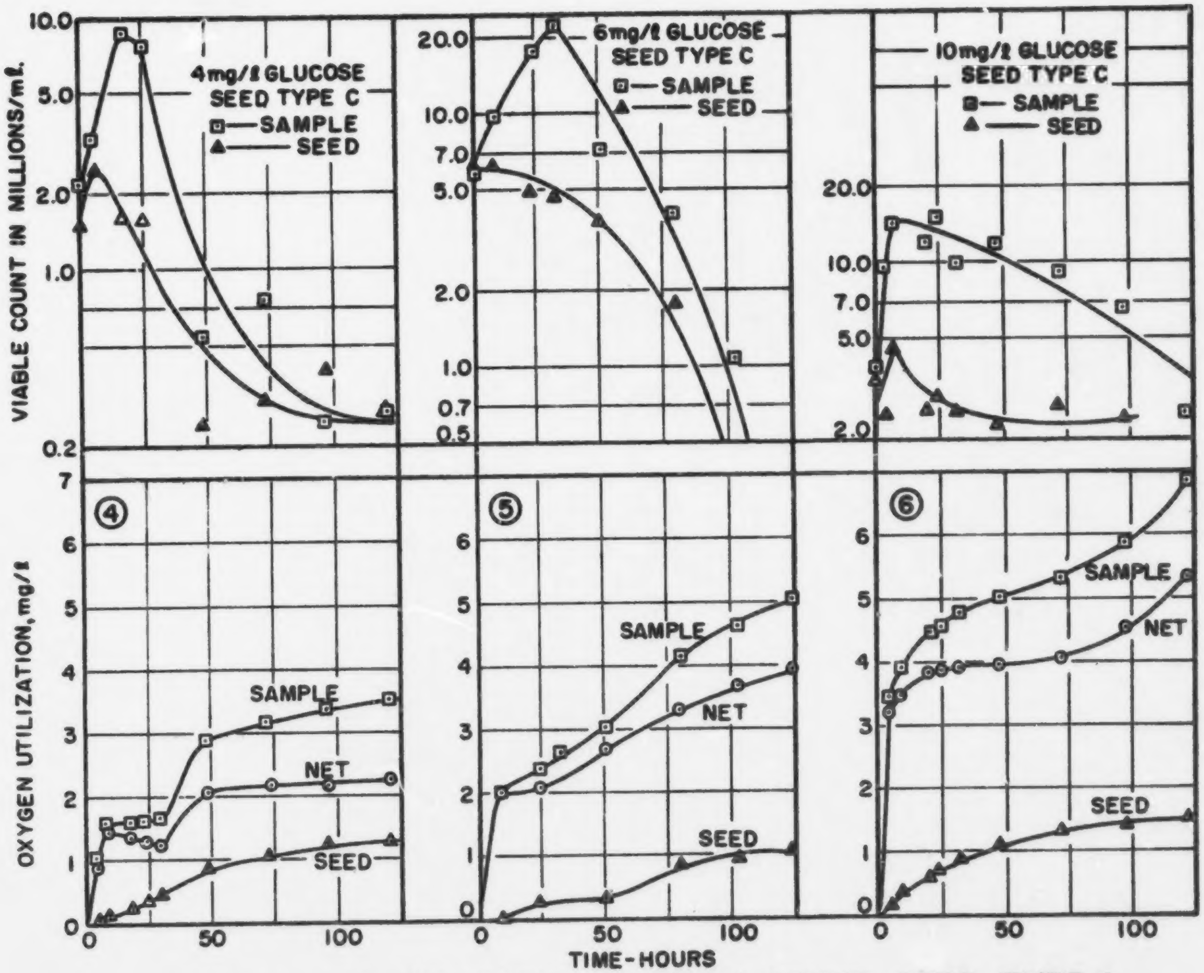

Fig. 4 - BOD AND VIABLE BACTERIA COUNT, SEED TYPE C

veloped from effluent of the laboratory activated sludge unit. Experiments are shown for glucose concentrations of 4, 6, and 10 mg/l. The plateau is evidenced in all experiments. It was so pronounced in the experiment shown at the left of the figure that subtraction of the seed yielded an apparent decrease in accumulated oxygen uptake in the sample bottle. In all cases the plateau occurred in the region of maximum population, and the last stage of BOD exertion corresponded to a dieoff in the viable population. In Fig. 5 the curves are extended to 480 hours. This figure shows the same general trend as found for the experiments plotted in Fig. 4. The seed system in this experiment, as with one of the systems shown in Fig..4, exhibits a multiphase oxygen uptake somewhat similar to that found in the sample systems.

Seed Type D

The results of five experiments using a pure culture of *Escherichia coli* which was grown, harvested and washed as for seed type C are shown in Fig. 6. In this series of experiments two substrate concentrations, 4 and 8 mg/l glucose, were used and the initial seed inoculum was maintained above 10^6 bacteria per ml. All experiments yield evidence of a plateau in oxygen uptake. In three experiments multiphase oxygen uptake was also observed for the seed system.

In the figure showing both 4 and 8 mg/l systems it is seen that the plateau is of longer duration in the system with lower substrate concentration. It is also interesting to note the dependence of oxygen uptake rate during the first stage of oxygen utilization upon substrate concentration. The system shown on the far right yielded a BOD curve somewhat different from the others shown

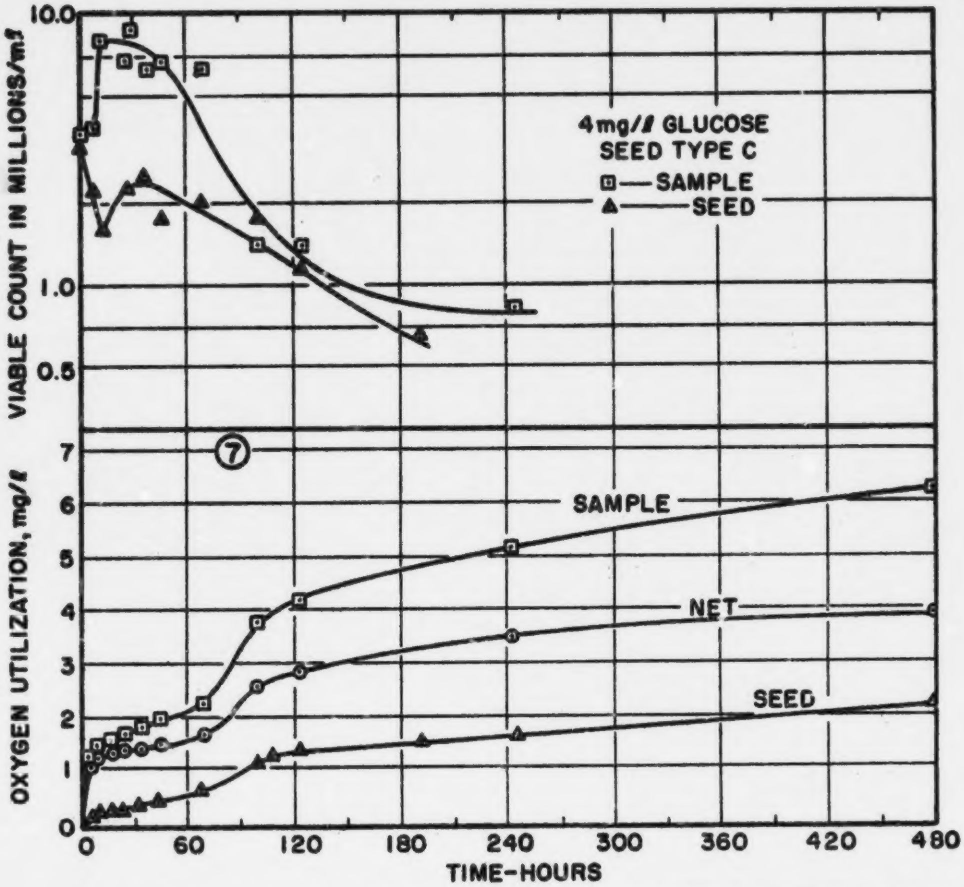

Fig. 5-BOD AND VIABLE BACTERIA COUNT, SEED TYPE C

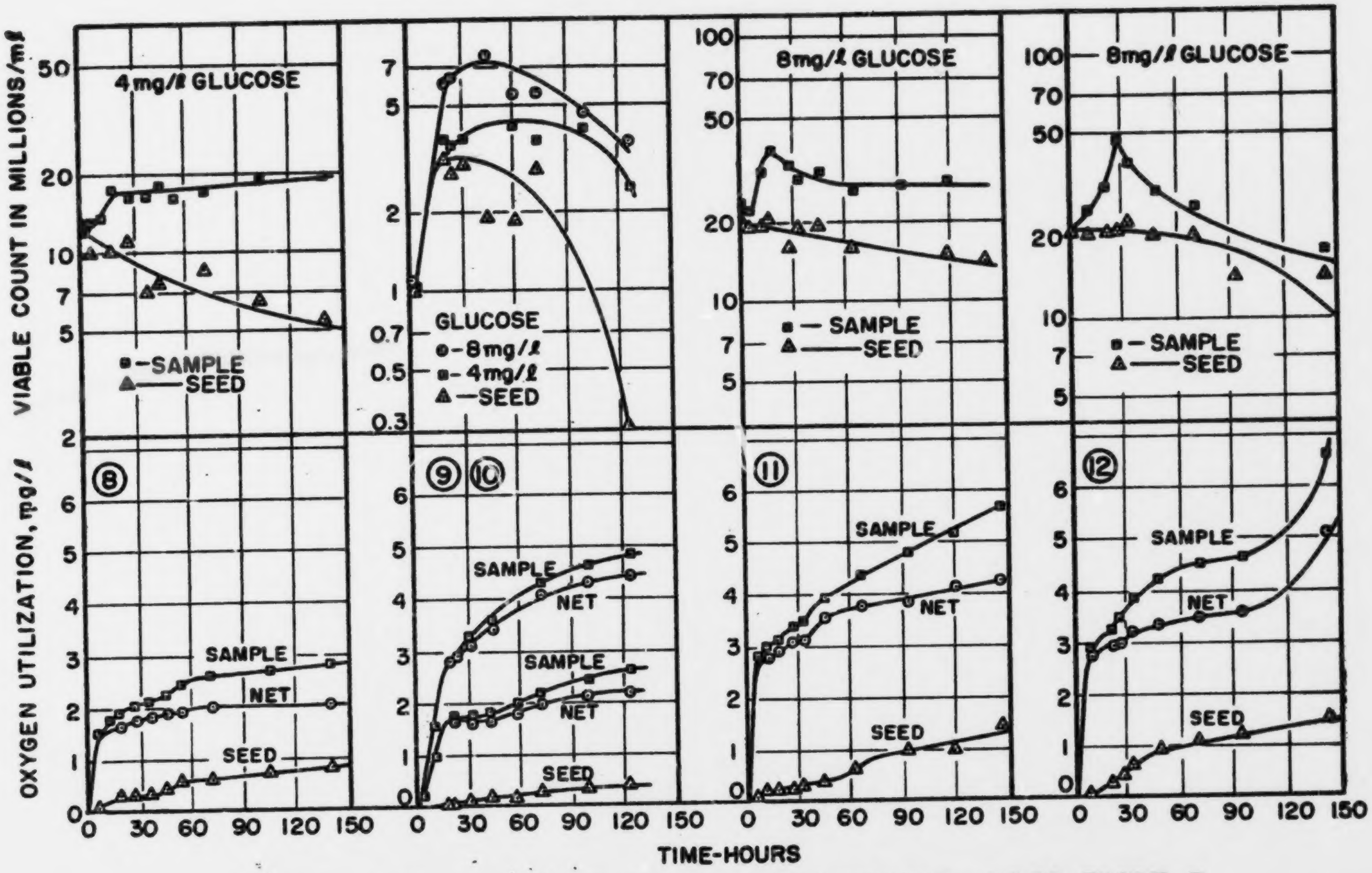

Fig. 6—BOD AND VIABLE BACTERIA COUNT, SEED TYPE D

in Fig. 6. There would appear to be two plateaus, one occurring in the usual time, which is followed by a decreasing rate portion, which then changes to an autocatalytic curve further along the time scale. Dissolved oxygen determinations after the 150th hour (not shown) indicated a rapid flattening of the curve and eventual net O_2 uptake of approximately 75% of the theoretical demand. For most experiments herein presented samples were incubated out to twenty days, but extended oxygen uptake is not shown in order to conserve space. In all such cases the O_2 uptake curves eventually approximated the typical first order decreasing rate curve.

For the experiments shown in Fig. 7, the Escherichia coli seed was used to compare results at high and low initial seed populations. In both cases a plateau was observed. It is seen that at the low seed concentration, even though the seed was washed three times, enough substrate was carried over to provide for approximately six doublings of the population. During this time there was no discernible oxygen uptake as measured by the Winkler method. Oxygen uptake in the seed did not occur until the dieoff period was under way. The plateau in the BOD exertion curve again occurred in the range of maximum viable population.

The rise in viable population in the seed deserves further discussion. Such a rise is sometimes noted when using low initial seed populations. In experiment 13 there was an increase in viable population of approximately 500,000 cells. Part of the increase might be due to a change in cell volume, but it is difficult to envision how the parent cells could store enough cellular material to provide for more than one doubling. Substrate carryover could account for some of the population increase. These cells were

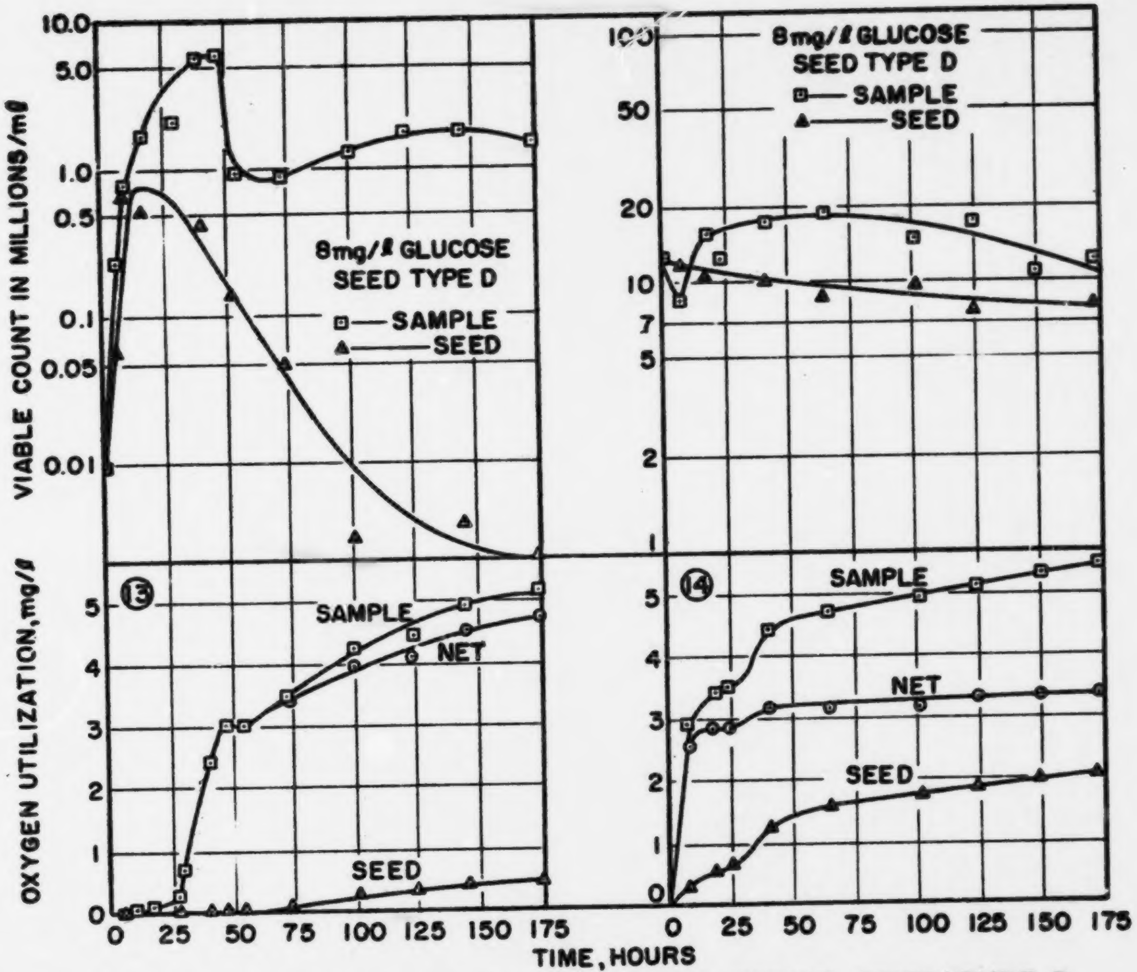

Fig. 7 - BOD AND VIABLE BACTERIA COUNT, SEED TYPE D

harvested for seed during the log growth phase from a medium in which the initial glucose concentration was 5000 mg/l. A considerable amount of substrate may have been left in the medium at the time of harvesting; however, three centrifugation and washing cyles should have reduced carryover to negligible concentration. It is interesting to calculate the approximate glucose concentration required to produce 500,000 cells. On the average the dry weight of a bacterial cell (coccus or a normal rod) may be taken as $2\text{x}10^{-10}$ milligrams, and assuming that 50% of the weight of substrate removed is used for respiration, the following calculation can be made:

Substrate channelled into)
synthesis to produce)= $2\text{x}10^{-10}$ mg/cell x $0.5\text{x}10^{6}$ cells/ml
$0.5\text{x}10^{6}$ cells) = $1\text{x}10^{-4}$ mg/ml
= 0.1 mg/l

Including substrate respired, the food concentration required would be 0.2 mg/l, which is a fairly small concentration. However, it is extremely doubtful that this amount could be carried over. Also, using the above basis of calculation, it is seen that O_2 uptake of approximately 0.1 falls within an acceptable range of error for D.O. measured by the Winkler method.

Another possible explanation is that the rise in population was due to "declumping." Although there was no discernible clumping or chain formation, and every effort was made to thoroughly disperse the cells, it is quite impossible to say that some cell aggregates did not exist in the initial seed inoculum. In addition it is possible that there were also dead cells in the initial inoculum which after lysis could provide food in addition to carryover substrate. Since all of these possibilities involve extremely low amounts of substrate, their relatively minor effect is not in evidence in the sample systems or when using high initial seed concentrations.

The plateau was also observed for the high seed concentration; however, the second stage net oxygen uptake curve was not as pronounced as for the system with lower initial viable count. Oxygen uptake in the seed was very high and was observed to be multiphased, as found for some of the seed curves shown in Fig. 6. The percentage of the theoretical oxygen demand of the substrate exerted up to the plateau for all experiments is given in Table I. This parameter varied from 30 to 38%, with an average of 34.0 for the fourteen experiments presented.

DISCUSSION

The results herein presented show with reasonable certainty that the plateau in oxygen uptake during exertion of carbonaceous BOD exists, using various seed materials and glucose as the carbon source. The phenomenon would not appear to be specific for glucose, since other workers have demonstrated the plateau using phthalic acid(8) and other pure compounds such as lactose, glutamate, acetate, propionate, and aspartic acid.(13) A slight plateau has also been found by Wilson and Harrison(8) for various plant effluents, although their figures leave some doubt as to its identity with the type of phenomenon demonstrated by Busch(7), McWhorter and Heukelekian(9), and that herein presented. In the latter three cases the plateau is so pronounced that the curve of BOD exertion cannot adequately be treated using any of the present kinetic equations which attempt to describe the entire course of carbonaceous BOD exertion; herein lies one of the major points of significance concerning the occurrence of the plateau. While it may be possible,

Table I

PERCENTAGE OF THEORETICAL OXYGEN DEMAND EXPRESSED AT THE PLATEAU IN THE BOD CURVE

Experiment Number	Theoretical Demand mg/l	Demand at Plateau	% Theor.	Remarks
1	8.5	2.6	31	Fig. 2
2	5.3	2.0	38	"
3	9.6	3.2	33	Fig. 3
4	4.3	1.5	35	Fig. 4
5	6.4	2.0	31	"
6	10.7	3.9	37	"
7	4.3	1.3	30	Fig. 5
8	4.3	1.5	35	Fig. 6
9	8.5	2.9	34	"
10	4.3	1.6	37	"
11	8.5	2.7	32	"
12	8.5	2.8	33	"
13	8.5	3.0	35	Fig. 7
14	8.5	2.8	35	"
			(34 Avg)	

as suggested by Busch(7), to use the plateau value (percentage of theoretical O_2 uptake) to predict total carbonaceous BOD, this does not allow adequate prediction of the kinetics of the entire BOD curve since, as seen in the results herein presented, the shape and length of the plateau region are not constant. In some cases the plateau represents only a short pause in O_2 uptake followed by either autocatalytic or decreasing rate kinetics while in other cases it is a long pause. In some cases oxygen uptake comes to a hault in the plateau region, while in others the plateau represents more of a flattening in the curve. In some cases there is a plateau in the seed system as well as in the sample system. The plateau in the seed occurs nearly at the same time as in the sample systems. Furthermore, the plateau in the seed may mask the plateau in the sample as shown in experiment 8 (Fig. 6). In this case net oxygen uptake (BOD) was diphasic, but had no plateau.

Another major significance of the occurrence of the plateau lies in the study of mechanism(s) which cause its occurrence. One of the prevalent theories of waste purification states that rapid oxygen uptake represents incorporation of all substrate into storage products and/or new cells, and the last phase represents endogenous respiration of stored products. If this theory holds in dilute suspensions, the dieoff of the bacterial population observed in our experiments could be interpreted as dieoff only the sense that the cells are no longer capable of replication but can still respire. In systems where protozoa are known to exist, a bacterial dieoff accompanied by a second stage of oxygen uptake can be partially attributed to ingestion of bacteria as food for the protozoa. In the work herein presented, the plateau was not as pronounced for pure culture systems as it was in heterogeneous systems. Therefore, based upon work using this particular pure culture, it cannot be concluded that masking of the plateau is due to protozoa, nor can it be concluded, as suggested by Busch(7), that they are largely responsible for the second stage of O_2 uptake. In addition, the magnitude of oxygen used in the second phase does not, for our studies, appear to "obviously depend upon the number of cells present," as suggested by Wilson and Harrison.(8) Comparison of experiments 13 and 14 in Fig. 7 indicates that the magnitude of the second stage was greater in the system containing fewer viable bacteria.

The dieoff of cells occurs in both pure culture and heterogeneous systems, although for the pure culture herein used the dieoff was not as pronounced as for the three types of heterogeneous seed. The dieoff curve for any one pure culture of bacteria cannot be compared to the dieoff for a heterogeneous system, since some pure cultures do not show as rapid a dieoff curve as others. This may depend upon differences in the integrity of various species as the culture ages.

Other investigators have studied changes in bacterial population and oxygen uptake during the utilization of dilute organic carbon sources. Butterfield studied oxygen uptake and viable count, using B. aerogenes and a small sewage coccus.(14) For these particular pure cultures there was no appreciable dieoff of cells in the 160 hour experiment; also his data show no evidence of a plateau. The shape of the oxygen uptake curve was the same as the viable count curve.

Lea and Nichols have also followed the course of carbonaceous BOD exertion and viable count.(15) Daily sampling over a five day period showed that the bacterial count curve was essentially of the same shape as the BOD curve. The viable count increased steadily at a decreasing rate, reaching a maximum population at the close of the experiment, five days.

In both of the works cited above there was no evidence of a plateau in BOD exertion. More recently Lee and Oswald have traced the course of BOD exertion and changes in viable population(16). Their data show a rapid rise in bacterial count, with maximum population being reached in 1 to 1½ days. The oxygen uptake rate was also determined for two and six-hour intervals. These data show an extremely rapid uptake rate followed by a sharp decrease. The maximum rate occurred during the first day; that is, slightly before the peak in viable count. No plateau is indicated in their accumulated uptake curve (corrected for lag period). However, the rate curve shown in one of the two experiments presented would seem to indicate a rapid rise followed by a plateau and a second stage of O_2 uptake. The rapid phase of oxygen uptake was attributed to cell growth. However, they considered it probable that although cell division took place, the cells remained in clumps or flocculent masses and therefore the recorded count would be less than the actual count. A rise in cell count was attributed to either "swarming" or mechanical or chemical dispersion of the clumps. For all studies herein reported, except those using seed type A, every effort was made to use non-flocculated cells, and we did not observe the lag in count compared to rapid oxygen uptake. If flocculated seed was used by Lee and Oswald, this could explain why the apparent plateau which can be surmised from their data (Fig. 5) comes slightly before the range of maximum population.

From our studies there is a clear indication that: 1) the rapid rise in oxygen uptake, first stage, corresponds to rapid bacterial multiplication; 2) the plateau corresponds to the peak range of population density, stationary phase; 3) the second stage of O_2 uptake corresponds to a decreasing portion of the bacterial count curve. This does not indicate that a rapid O_2 uptake must be due to multiplication of cells, since rapid synthesis can occur in systems which militate against cell multiplication at almost the same rate as in systems which promote cellular replication. However, in the BOD bottle nitrogen is present in ample supply (unless the substrate or waste concentration is so high that the nitrogen concentration becomes the limiting growth factor, in which case the total course of BOD exertion cannot be traced before depletion of dissolved oxygen), and the energy derived from the respiration process is readily channelled into synthesis leading to cell multiplication.

Regardless of the clear demonstration that the rapid phase, plateau and second stage in carbonaceous BOD exertion can correspond to rapid growth, peak or stationary phase and the dieoff phase in viable population, this does not explain the cause of the occurrence of the plateau. Based upon this work and other work we have accomplished in such dilute systems as that prevailing in the BOD bottle and work in high energy systems, it is possible to predict causative mechanism. These are presented and discussed in the succeeding paper(17).

ACKNOWLEDGEMENT

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PAPER 5

STUDIES ON THE OCCURRENCE OF THE PLATEAU IN BOD EXERTION. II. EXPERIMENTS USING DIFFERENT CARBOHYDRATES AND MULTICOMPONENT SYSTEMS

By

A. F. Gaudy, Jr. and M. N. Bhatla

See note, Paper 4.

STUDIES ON THE OCCURRENCE OF THE PLATEAU IN BOD EXERTION II. EXPERIMENTS USING DIFFERENT CARBOHYDRATES AND MULTICOMPONENT SYSTEMS

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A preceding paper (1) described investigations of the occurrence of the plateau in BOD exertion, using both heterogeneous and pure culture seed, and glucose as carbon source. It was found that the plateau was of general occurrence and that the first stage of O_2 uptake corresponded to a rapid rise in the viable population (except in cases where a fairly high initial seed population was employed which precluded such a rise at the food concentrations prevailing in the BOD bottle). The plateau occurred at maximum population density, and the second stage in oxygen utilization corresponded, in general, to a drop or dieoff in the viable population. While the correlation of changes in O_2 uptake rate with various phases of population dynamics in the BOD test was generally observed, it does not appear necessary that such a correlation exist in order for the plateau to occur. However, it is felt that a decrease in viable population may contribute to the production of a plateau. Four somewhat related theories of causation for the plateau are presented and discussed in the concluding section of this report.

The results herein presented are an extension of the work to a variety of compounds using young heterogeneous populations acclimated to the particular compounds under study (seed type C) and multicomponent systems using both young heterogeneous populations (seed type C) and a pure culture (seed type D). This work was accomplished in an effort to gain a further insight into the general occurrence and causation of the plateau. The techniques used were those previously described (1). Where further explanation is desirable, for some of the experiments herein included, it is given as the results are presented.

Portions of this paper were presented at the 18th Industrial Waste Conference, Purdue University, May, 1963.

RESULTS

Occurrence of the Plateau for Various Compounds

In further effort to determine the generality of the occurrence of the plateau, BOD experiments were undertaken using seed grown and harvested in the same manner as for seed type C except that the cells were grown, not on glucose, but on the particular carbon source studied. Results of eight such experiments are given in Fig. 1. In order to present all eight experiments in one figure, thus facillitating comparison of the systems, it was impossible to show oxygen uptake and population dynamics for the seed; therefore, only net O_2 uptake and the viable count in the sample bottles are shown. Results pertinent to the seed systems are given in the text. Viable count for the glycerol system (Experiment 1, Fig. 1) was not obtained.

It is seen that there was definite evidence of a plateau in net oxygen uptake for all systems except glycogen and galactose (Experiments 2 and 8); however, the total oxygen uptake curve for these experiments (not plotted) showed a slight plateau which corresponded to one in the seed. Subtraction of the oxygen uptake of the seed yielded a net curve with no plateau in the glycogen system, whereas for galactose the second stage uptake in the seed was more abrupt and upon subtraction yielded a net curve which shows a negative oxygen utilization (see last three points Experiment 8). Oxygen uptake of the seed for lactose, ribose, glutamate, sorbitol and melibiose was also diphasic but did not mask the plateau in net oxygen uptake. The total uptake for glycerol gave only a slight indication of a plateau, but the seed uptake broke so sharply that it accentuated the plateau in net oxygen utilization. Diphasic oxygen uptake in the seed was also noted for some of the glucose experiments previously presented (1).

Although the plateau in BOD exertion occurs with and without a similar diphasic occurrence in the seed, it would seem that they are logically related, and since occurrences in the seed are observed to affect the shape of the net curve it would appear that possible attempts to hasten the course of BOD expression by increasing the seed inoculum should be approached with caution. It would appear at first glance better to use a very low initial seed and accept an autocatalytic curve as the standard BOD curve. How-

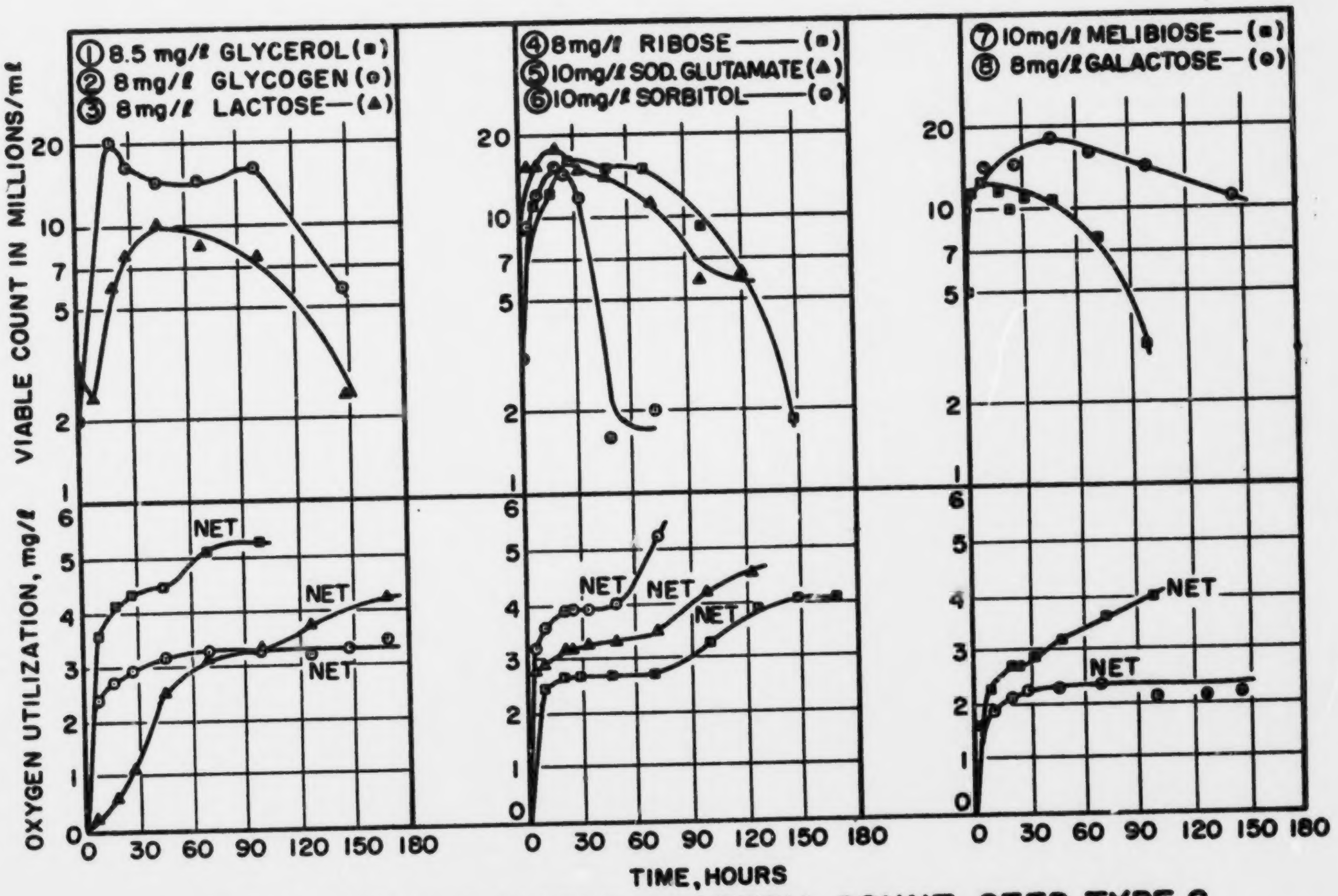

Fig. 1– BOD AND VIABLE BACTERIA COUNT, SEED TYPE C

ever, this would have serious ramifications to the interpretation of the standard five-day BOD test unless the Thomas lag concept can be routinely applied. Also this would not obviate need to consider the plateau; the only advantage that would accrue is that phenomena in the seed would be less felt in net uptake and the shape assumed by the BOD curve would be more completely attributable to the substrate or waste sample. The viable count curves for all seed systems were of the same general shape as the curves shown in Fig. 1 for the sample flasks. A slight rise in the seed count of approximately 1/10th of the rise in the sample systems was followed by a dieoff rate not quite as sharp as that exhibited in the sample bottles.

In all experiments shown in Fig. 1 which exhibit a plateau it is seen that, in general, the plateau region corresponds to the region of maximum population density. The longer the pause in viable count before the dieoff, the longer is the plateau. The second stage uptake in general corresponds to the region of dieoff in viable count.

Studies on Multicomponent Systems

Substrate removal in multicomponent synthetic waste systems is currently under investigation in our laboratory. Most of this work is conducted at high energy levels, i.e., at substrate concentrations approximately 100 times greater than those used in the BOD bottle. In such systems sequential removal of the carbon sources has often been found to be reflected in diphasic oxygen uptake curves. If such were the case in low energy systems, this might cause a discernible plateau in the BOD curve. Accordingly experiments using multicomponent carbon sources were undertaken.

Glucose plus Ribose, Seed Acclimated to Ribose

Figure 2 shows the course of BOD exertion and viable count for a synthetic waste consisting of 4 mg/l glucose plus 4 mg/l ribose. Control systems for glucose and ribose are also shown. The seed was grown on glucose and harvested according to the procedure described for seed type C. All systems, including the seed, exhibited phasic oxygen uptake. Acclimation to ribose took place in less than six hours as seen from the ribose control sys-

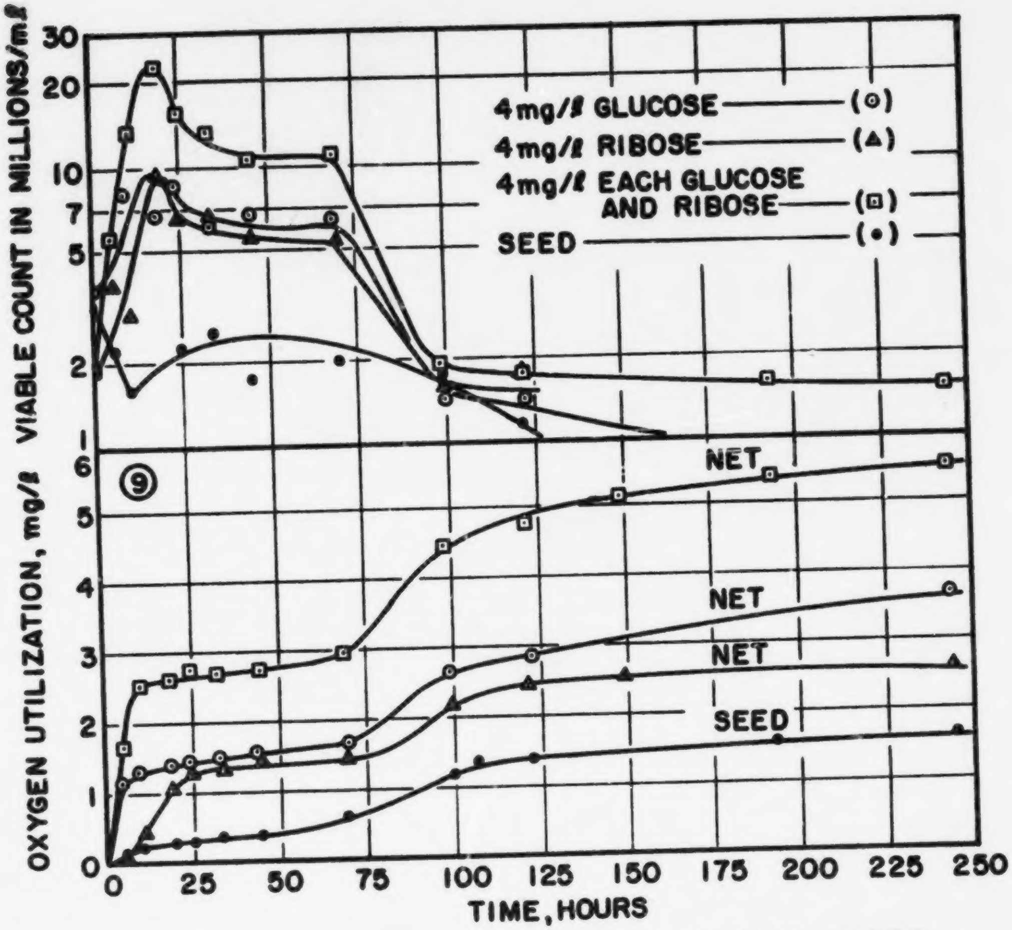

Fig. 2 - BOD AND VIABLE BACTERIA COUNT, SEED TYPE C ACCLIMATED TO GLUCOSE.

tem, which also shows a plateau at approximately 25 hours. The glucose control and the combined system also exhibited a plateau which occurred at approximately 10 hours. From these results it does not seem that the observed plateau was related to sequential removal of glucose and ribose, since if it were it would not be expected that oxygen utilization in the combined system would continue to increase after the initial break in the glucose control. Furthermore, a plateau was observed in all systems. It would seem that any diphasic nature of the oxygen uptake curve due to sequential uptake, first on glucose and then on ribose, would have had to occur before the extended plateau which was observed. Also acclimation to ribose was fairly rapid; therefore a plateau caused by sequential removal would not have been observed, since the first sample was withdrawn at 6 hours incubation. It was also observed that the oxygen uptake of the seed was diphasic, with the second phase corresponding to that in the sample systems.

Glucose plus Sorbitol, Seed Acclimated to Glucose

Figure 3 shows results of an experiment using glucose and sorbitol as the multicomponent carbon sources with seed obtained in the same manner as in the previous experiment (seed type C). The seed did not exhibit diphasic oxygen uptake, but the controls and the combined substrate system did. The results, in general, are similar to the previous experiment; however, the plateau is much shorter. Comparison of Figures 2 and 3 indicates that the length of the plateau is correlated to the sharpness of the peak in viable population.

Glucose plus Sorbitol, Seed Acclimated to Sorbitol

Figure 4 shows results in a system similar to that shown in Figure 3, but the cells were acclimated to sorbitol instead of glucose. The control systems exhibited a slight plateau; the combined substrate system is diphasic, with only a slight indication of a plateau. As in the previous experiment, the growth and dieoff patterns of the viable population are related to the plateau.

Glucose plus Sorbitol, Pure Culture Seed (Type D Acclimated to Glucose)

Figure 5 shows results of two experiments using glucose-sorbitol carbon sources and Escherichia coli (seed type D) accli-

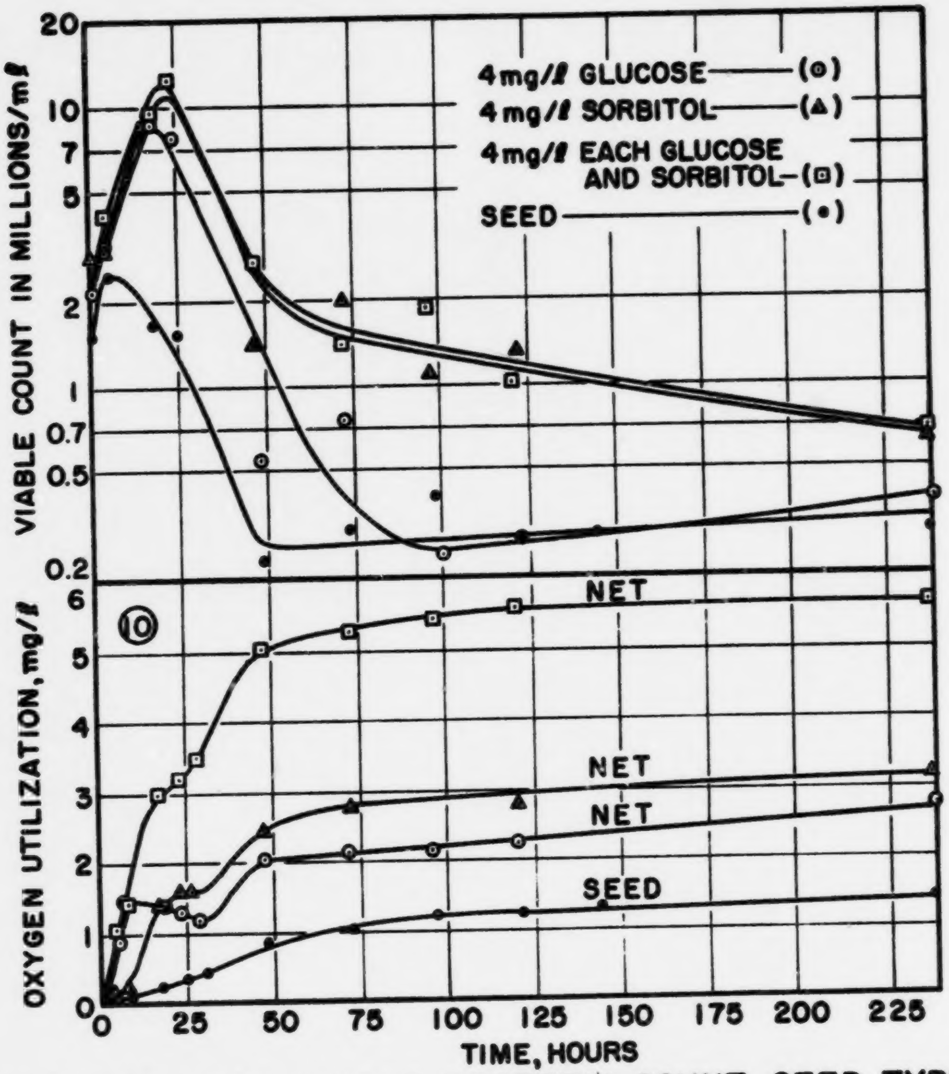

Fig. 3 – BOD AND VIABLE BACTERIA COUNT, SEED TYPE C ACCLIMATED TO GLUCOSE.

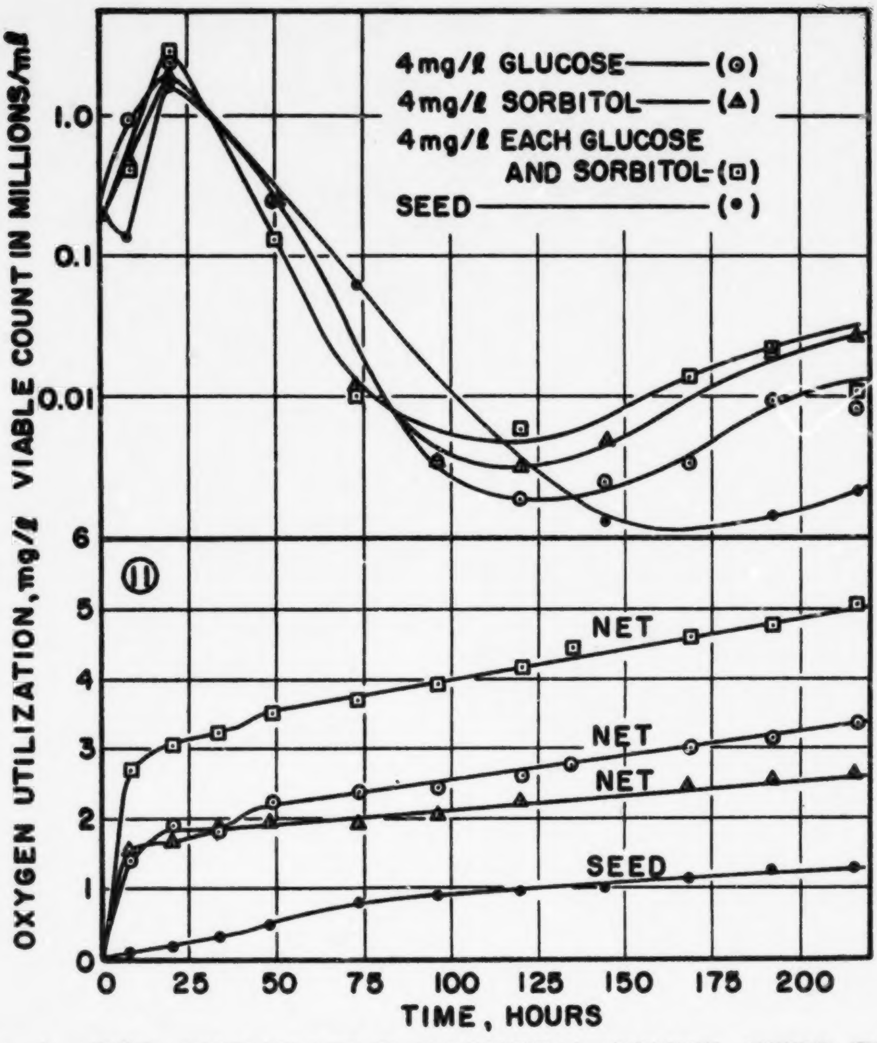

Fig. 4 - BOD AND VIABLE BACTERIA COUNT, SEED TYPE C ACCLIMATED TO SORBITOL.

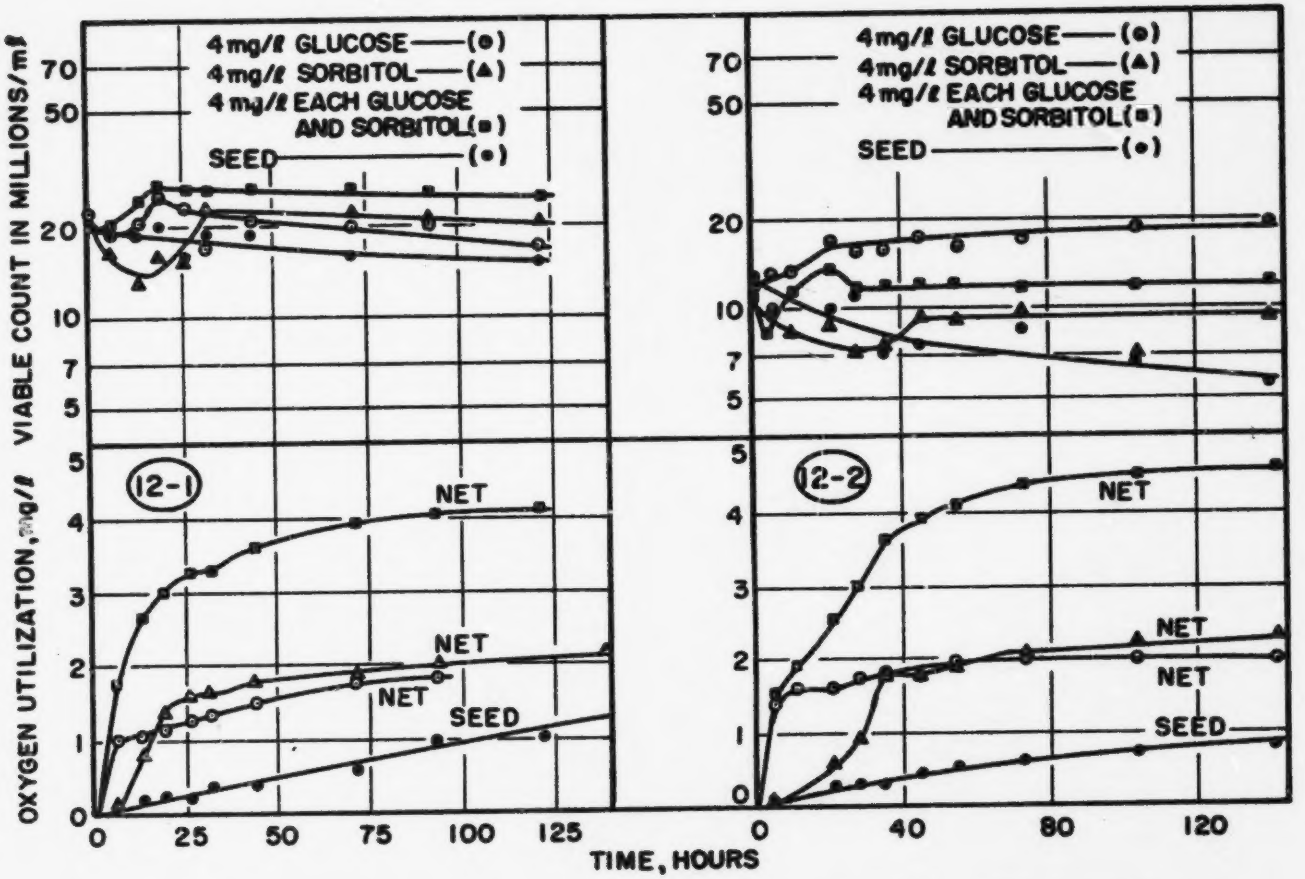

Fig. 5 — BOD AND VIABLE BACTERIA COUNT, SEED TYPE D ACCLIMATED TO GLUCOSE.

mated to glucose. In Experiment 1 the initial inoculum was approximately double that used in Experiment 2; both were above 10^7 cells per ml. In both experiments the controls (glucose and sorbitol) show diphasic oxygen uptake. Both combined systems exhibit a plateau but at different locations on the curve. Experiment 12-1 shows the same patterns previously found for heterogeneous populations (Figure 3), whereas the location of the plateau and the extent of the second stage of oxygen uptake for Experiment 12-2 suggests diphasic removal of the two exogenous carbon sources. The very high initial cell inocula used precluded an extensive rise in population. Also a multifold dieoff was not observed, whereas it was generally found for lower initial inocula (1). The percentage of the theoretical oxygen demand exerted at the plateau for all experiment is given in Table I.

DISCUSSION

From the results herein presented and those given previously (1), it is seen that a discernible plateau in the expression of carbonaceous BOD was evidenced in 34 of the 37 experiments. It has been shown to occur using sewage seed, laboratory pilot plant effluent, washed sludge suspensions, and a pure culture, prototrophic strain of Escherichia coli at various concentrations of initial seed. It has been shown to occur using a variety of substrates, and in various multicomponent systems. While these experiments attest to the generality of its occurrence and its general location at approximately 30-40% of the theoretical oxygen demand, little can be stated concerning its severity and duration, and thus its effect upon predictive evaluation of the kinetics of BOD exertion. Under "normal" conditions of seed and substrate concentrations in the BOD bottle, the first stage in oxygen uptake was found to correspond to a rising portion of the viable population curve, the plateau to the region of maximum cell density, and the second stage of O_2 uptake to a decrease or dieoff portion of the viable count curve. This should not be taken to indicate that diphasic oxygen uptake or the occurrence of the plateau is restricted to growth systems; it could conceivably occur under nonproliferating conditions as well. This particular aspect is worthy of further

TABLE I

Percentage of Theoretical Oxygen Demand Expressed at the Plateau in the BOD Curve

Exp. No.	Theoretical Demand, mg/l	Demand at Plateau mg/l	% Theoretical	Remarks
1	10.4	4.5	43	Fig. 1 (Glycerol)
2	8.05	3.2	38	" (Glycogen)
3	8.5	3.3	38	" (Lactose)
4	8.5	2.7	32	" (Ribose)
5	8.0	3.2	40	" (Sod.Glutamate)
6	11.4	3.9	34	" (Sorbitol)
7	10.7	2.7	25	" (Melibiose)
8	8.5	2.3	27	" (Galactose)
9	4.3	1.4	33	Fig. 2 (Glucose)
	4.3	1.3	30	" (Ribose)
	8.5	2.6	30	" (Gl. + Rib.)
10	4.3	1.4	34	Fig. 3 (Glucose)
	4.6	1.6	35	" (Sorbitol)
	8.8	3.2	36	" (Gl. + Sorb.)
11	4.3	1.8	43	Fig. 4 (Glucose)
	4.6	1.6	36	" (Sorbitol)
	8.8	3.1	35	" (Gl. + Sorb.)
12-1	4.3	1.1	26	Fig. 5 (Glucose)
	4.6	1.6	35	" (Sorbitol)
	8.8	3.3	37	" (Gl. + Sorb.)
12-2	4.3	1.6	37	" (Glucose)
	4.6	1.8	40	" (Sorbitol)
	8.8	3.6	41	" (Gl. + Sorb.)

investigation. It is emphasized, however, that in the standard BOD test growth conditions normally obtain. As seen in many of the experiments, a plateau may also exist under endogenous conditions (seed blank).

Of perhaps more immediate importance than the effect of the plateau on formulations for the prediction of BOD kinetics are explanations of causation for the existence of the plateau. The plateau shown in Figure 5 (Experiment 12-2) for the glucose-sorbitol medium could, in light of the curves for the glucose and sorbitol controls, be attributed to sequential substrate removal. Although this may be a valid cause for the existence of a plateau, it cannot be cited as the general mechanism since the plateau occurs with single exogenous carbon sources as well, and at times also in endogenous systems which are purposely held devoid of exogenous carbon source (washed seed blanks).

While it is relatively easy to cite possible explanations for the various shapes that the BOD curve may assume, basic explanation(s) for the plateau, or pause between phases, is more difficult and requires somewhat more basic biochemical considerations. Indeed, there may be various ways in which the plateau may be produced. Based upon the results herein presented and those we have obtained in high substrate concentration systems, the following possibilities seem prominent:

(1) In a heterogeneous cell system, after reaching the stage of maximum growth (cell production or combination of cell production and storage), the predominating cells begin to die or otherwise lose their turgor and release cellular components which may become food for other species of cells. Since these were not the predominating species, time is required before they can grow to such numbers that their growth is expressed in oxygen uptake. This might lead to two autocatalytic curves; the end of the first phase and the beginning of the second form the plateau.

(2) The phenomenon cited above occurs without the aftergrowth of a second species; that is, there is no change in predominance, but because of the different structural configuration of the original substrate and the released cellular components, an acclimation period is required to synthesize the new enzymes needed to metabol-

ize the new, and now exogenous, substrates. If enough cellular components are released, an aftergrowth may occur; on the other hand, the released components may not be of sufficient quantity to support much new growth, and the dieoff in total population continues. It is also possible that the released components may provide enough energy to maintain the viable integrity of the cells, and cell dieoff proceeds more slowly.

Theories (1) and (2) differ only in that the second stage of O_2 uptake is caused by a new predominance in the population in theory (1), and by the same population with a required acclimation period in theory (2). The fact that the cells themselves may become food for protozoa would seem to form an incidental part of theory (1). The fact that the plateau is short in some cases and long in others can arise by a combination of (a) time for acclimation, and (b) time to gain enough population to be expressed in oxygen uptake.

(3) Cells do not die in the sense that they no longer can respire, but they may lose their ability to replicate. An acclimation period is needed before cellular components formerly synthesized in replicating processes can be broken down to supply energy. Another possibility within the scope of theory (3) is that an acclimation period is needed before the cells can use certain "storage products" for energy. In this case the length of the plateau, and indeed its existence in such systems, would depend upon the nature of the storage products and the induction time, if required, for producing the necessary catabolic enzymes. Reactions of this type might provide an explanation for the delayed onset of endogenous respiration suggested as a possible cause for the plateau by Wilson and Harrison (2).

(4) During rapid metabolism of some compounds the cells release intermediates into the media rather than channelling all such compounds into storage products. In such cases time may be required for induction of enzymes to initiate metabolism of the new exogenous compounds. Time for the induction of transport catalysts may also be required to bring these new components to existing enzyme systems within the cell. Since there is no longer enough substrate to support continued growth, the cells begin to die off.

It is entirely possible that all four theories contribute to causing the plateau. Theory (1) is not generally applicable, since the plateau occurs using pure cultures. At lower initial seed populations the second stage was always accompanied by a fairly rapid dieoff in viable count. This parameter of internal system response measures only cells capable of replication. Although nutrient agar is a more complete medium than that generally present in the BOD bottle, and therefore should tend to favor growth of all cells capable of replication, some cells which are not viable may be capable of respiration. The BOD test itself is one which measures only respiration; the fact that cell replication can often be correlated with O_2 uptake is incidental to the test. The fact that some cells lost viability does suggest the existence of structural damage and the loss of synthesized components to the medium. If this is true, it would be expected than an indication or acclimation period would be required before the remaining intact cells could extract energy (registered as O_2 uptake) from the breakdown of cellular components. If cellular materials are not lost to the medium after the period of rapid cellular multiplication, as suggested in theory (3), an acclimation period could still logically be expected for the same reasons cited above. If, as stated in theory (4), metabolic intermediates are excreted during the rapid growth phase, an acclimation period which could be expressed as a plateau may be required before these can be metabolized. Even though in some cases the cells may already possess the enzymes required to metabolize the excreted intermediates, new enzyme(s) may have to be induced in order to transport these components back into the cell and to the site of enzyme action. Since most of the original energy-yielding external carbon source would have been depleted by the time the intermediates would be called upon, there would be a paucity of readily available energy source to synthesize the new enzyme(s), thus slowing down their production and leading to a lag or plateau between oxygen uptake phases.

It may be discerned that although all four theories differ in essential components, there is one central likeness. The plateau is caused by a change from metabolism of the original exogenous carbon source(s) to metabolism of new carbon source(s) produced by the cells. These may be excreted after maximum population is reached or during rapid metabolism or may remain in the cells, but the nature of the carbon source the cells are required to metabolize changes. This may or may not lead to changes in species predominance. In any event, when the change in carbon source is abrupt and an induction period is required, a plateau can occur. Work in this area is continuing in both high and low energy systems, with the primary aim of seeking general or unifying theories of causation for phasic phenomena during the course of waste removal.

SUMMARY AND CONCLUSIONS

1. The occurrence of a plateau in BOD exertion, according to the results herein presented, would appear to be independent of the type of seed employed.

2. In general, under "normal" seeding conditions and substrate concentrations in the BOD bottle, the first phase of oxygen uptake corresponds to a period of rapid cellular multiplication, the plateau occurrence to the maximum range of population density, and the second stage of oxygen uptake to a decrease in the viable population.

3. The duration or severity of the plateau corresponds approximately to the length of the stationary phase in the region of maximum population.

4. Four related theories explaining the existence of the plateau were discussed. These theories are similar in the sense that the plateau is caused by a change from metabolism of the original exogenous carbon source to the metabolism of the new carbon sources produced by the cells. The plateau comes about because of the need of an induction period before the new energy source(s) can be metabolized to such an extent that this metabolism is expressed in oxygen uptake.

ACKNOWLEDGEMENT

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PAPER 6

STUDIES ON THE OCCURRENCE OF THE PLATEAU IN BOD EXERTION

By

A. F. Gaudy, Jr., M. N. Bhatla, and F. Abu-Niaaj

This paper constains portions of the results from Paper 4 and Paper 5 and was presented at the Purdue Industrial Waste Conference, May, 1963. (See note, Paper 4).

STUDIES ON THE OCCURRENCE OF THE PLATEAU IN BOD EXERTION

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The BOD test has been the subject of continuing research and controversy since its inception during the latter part of the 19th century, and various formulations for predicting the course of carbonaceous BOD expression have been proposed. Figure 1 shows the various types of kinetics most prevalently observed. Curve 1 shows the "typical" first order decreasing rate curve usually used to predict the course of BOD exertion.(1) Much of the research on BOD has been devoted in recent years toward demonstrating that this kinetic curve does not suffice to depict the course of oxygen uptake in the BOD bottle. Often curve 2, which can be described as "autocatalytic", depicts the course of O_2 utilization. The lag correction of Thomas may sometimes be applied to adjust this curve to fit the kinetic order shown in curve 1.(2) The lag may follow first order increasing rate kinetics and correspond to a log phase of bacterial growth. The course of oxygen uptake is sometimes described by a curve similar to curve 3 in which oxygen uptake may follow decreasing rate first order kinetics but one velocity constant does not describe the entire curve.(3)(4) More recently, BOD curves of

Presented at the 18th Industrial Waste Conference, Purdue University, May, 1963.

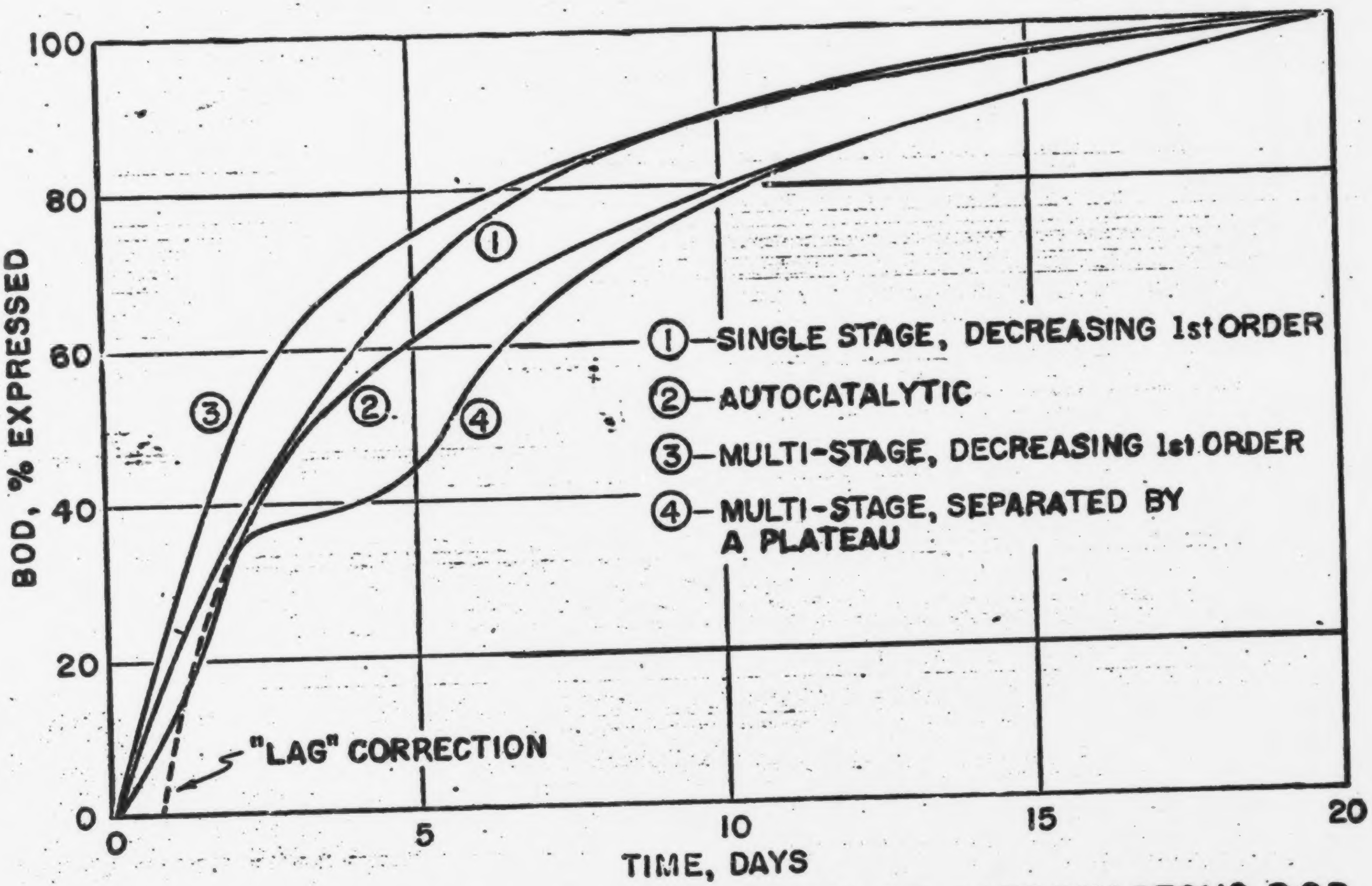

Fig. 1-VARIOUS KINETIC EXPRESSION OF CARBONACEOUS BOD [2]

type 4 have been observed in which a rapid first phase of oxygen uptake is separated from a second phase of uptake by a pause or plateau.(4)(5) It is the latter type of BOD curve which is the concern of this paper.

One of the present research interests in our laboratory is the study of the mechanism(s) and kinetics of substrate removal in activated sludge processes; these we term "high energy systems" (high biological solids and substrate concentrations) in contrast to low energy systems such as exist in the BOD bottle and usually in the receiving stream. However, one of the sanitary engineering parameters for measuring process efficiency which we would like to use is the BOD test. It was reasoned that if the plateau is of general occurrence, the standard BOD test would be of little or no value. It was therefore necessary to investigate the occurrence of the plateau in BOD exertion under various conditions which we would employ in our research. In undertaking such investigations we have been able to study discontinuous high and low energy systems concurrently. The major purpose of the investigation herein reported was to determine the generality and consistency of the occurrence of the plateau and to gain some insight into possible mechanisms of causation for its occurrence. The parameter used to gain such an insight was measurement of the change in viable bacteria count during the course of BOD expression.

MATERIALS AND METHODS

Throughout the investigations, BOD and dissolved oxygen reagents were those prescribed in _Standard Methods for the Examination_ of Water and Wastewater.(6) Dissolved oxygen was measured using the Alsterberg modification of the Winkler method. BOD's (seed blank and samples) were run using various substrates, types of seed, and seed concentrations. At each sampling time before adding Winkler reagents, 1 ml of each system was withdrawn, appropriately diluted, and used to determine the viable bacteria count. Preliminary experiments had shown that dissolved oxygen values were unaffected by the extra manipulation required in withdrawing the sample prior to determining the dissolved oxygen. Changes in population were measured in the seed blanks as well as in the sample bottles. This was done in order to obtain comparable information on both systems. In addition this procedure provided for a partial check on the carry-over of substrate with the seed.

The spot plate counting technique was used to estimate the viable bacteria count. This method, which involves the plating of a known volume of suspension on the surface of solidified agar, had been previously shown to yield as reliable and slightly more accurate population estimates for aerobic organisms than the standard pour plate technique.(7) The method has also been successfully employed in studies on the survival of Salmonella typhosa in high rate digesters.(8) In all studies herein reported, cells were plated on nutrient agar.

Four types of seeding material were used. Seed type A consisted of effluent from the primary clarifier of the municipal sewage treatment plant at Stillwater, Oklahoma.

Seed type B consisted of effluent from a batch operated laboratory activated sludge developed from an initial seed of type A and grown on the particular carbon source under investigation.

Young cells (seed type C) were developed from an initial inoculum of seed type B. They were grown on the particular carbon source under study. The cells were harvested by centrifugation near the end of the log growth phase and washed three times with 0.05M phosphate buffer.

Seed type D consisted of a pure culture, prototrophic strain, of Escherichia coli grown on a shaker apparatus, harvested near the end of the log growth phase and washed as in seed type C.

Seed and substrates were varied in concentration and type. Known multicomponent carbon sources were also investigated.

RESULTS

In all, 37 systems were examined for the occurrence of the plateau. Typical results are herein presented and discussed, and theories of causation for the plateau are given. More complete presentation of results and detailed analyses will be submitted elsewhere.

Seed Type A

Figure 2 shows the course of BOD exertion and changes in viable population for systems containing 8 and 5 mg/1 of glucose using municipal sewage seeds. Multiphase kinetics were exhibited in both experiments regardless of the initial concentration of viable cells. In both experiments there is definite evidence for the plateau in BOD exertion. The first or rapid phase of oxygen uptake corresponds to a log phase of growth. The plateau occurs in the region of maximum viable count, and the last stage of oxygen uptake corresponds to a sharp decrease in viable population. The behavior of the seed systems

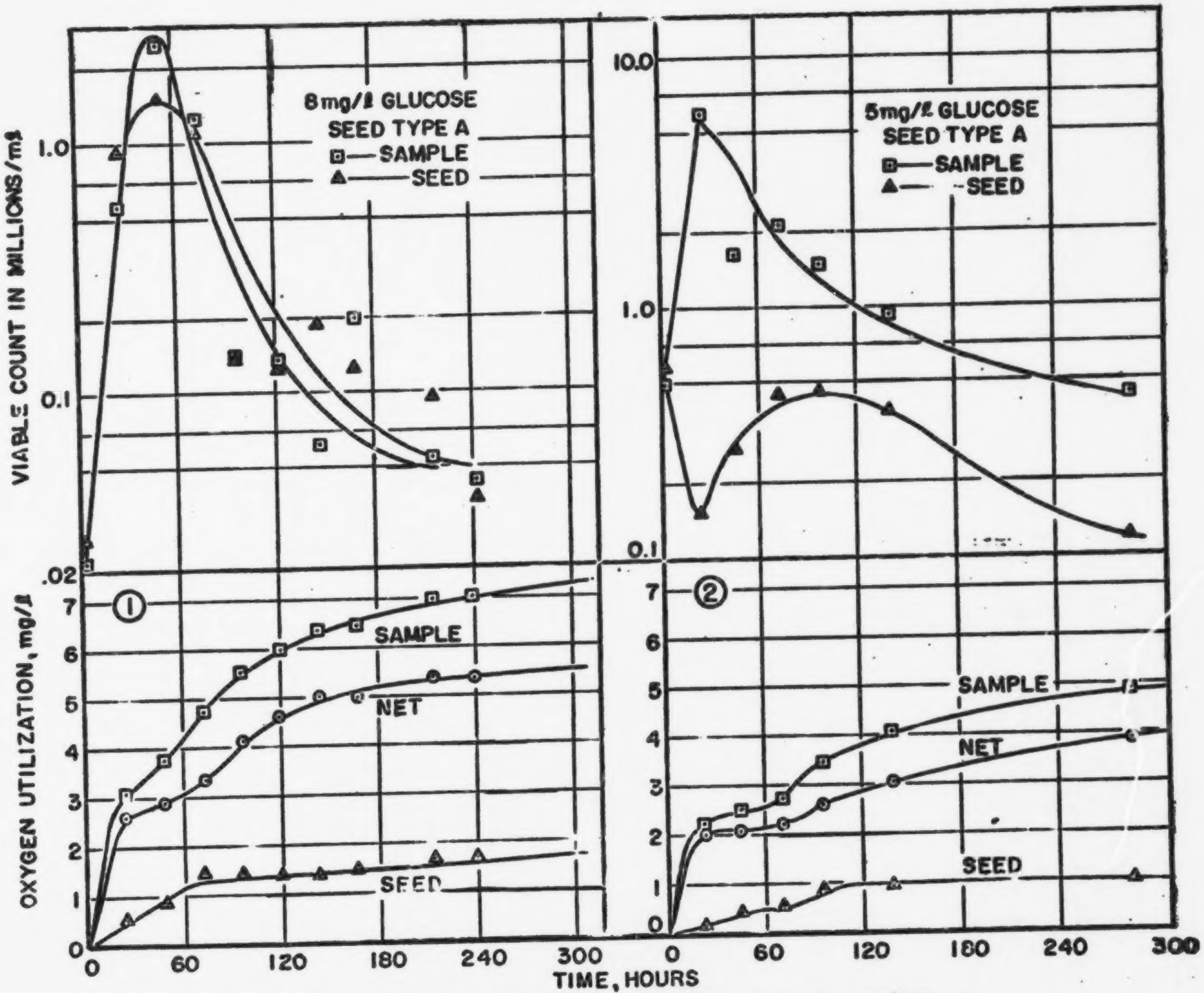

Fig. 2-BOD AND VIABLE BACTERIA COUNT, SEED TYPE A

is of particular interest. With a low initial inoculum (2×10^4) the viable count in the seed experiences a considerable log phase (approximately six doublings of the population). In the other experiment there is an initial decrease in viable cells followed by a slight increase, which in approximately 70 hours yielded a population density equal to that of the initial inoculum. The seed behavior may be attributed to the difference in population density in the seed, and to a variable strength of organic matter in the sewage from which the seed was obtained. Regardless of the variable nature of the seed, the plateau existed.

Seed Type B

Results for an experiment using seed obtained from the effluent of a laboratory activated sludge plant operating on glucose with sludge developed from an initial inoculum of municipal sewage are shown in Figure 3. In this case there was very little oxygen used in the seed system. The population remained static during the first 120 hours, then gradually fell off. The system containing 9 mg/l glucose shows the full gamut of BOD kinetics. There is a slight lag followed by an autocatalytic portion, both of which are reflected in the viable count data. The plateau is again in evidence, and corresponds to the range of maximum population density. The second phase of oxygen uptake again becomes autocatalytic and finally approaches the typical first order decreasing rate curve which has usually been cited as describing the BOD reaction.

Seed Type C

Figure 4 shows results using washed young heterogeneous seed developed from effluent of the laboratory activated sludge unit.

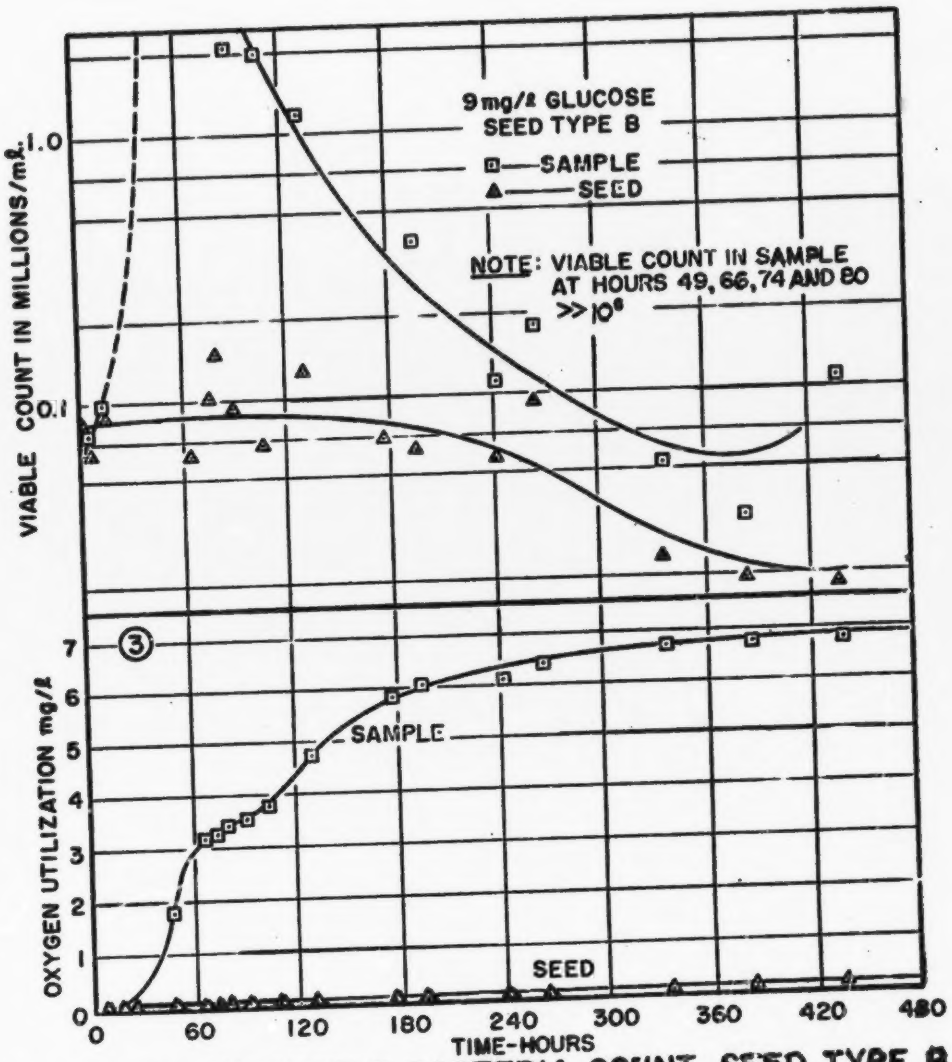

Fig.3-BOD AND VIABLE BACTERIA COUNT, SEED TYPE B

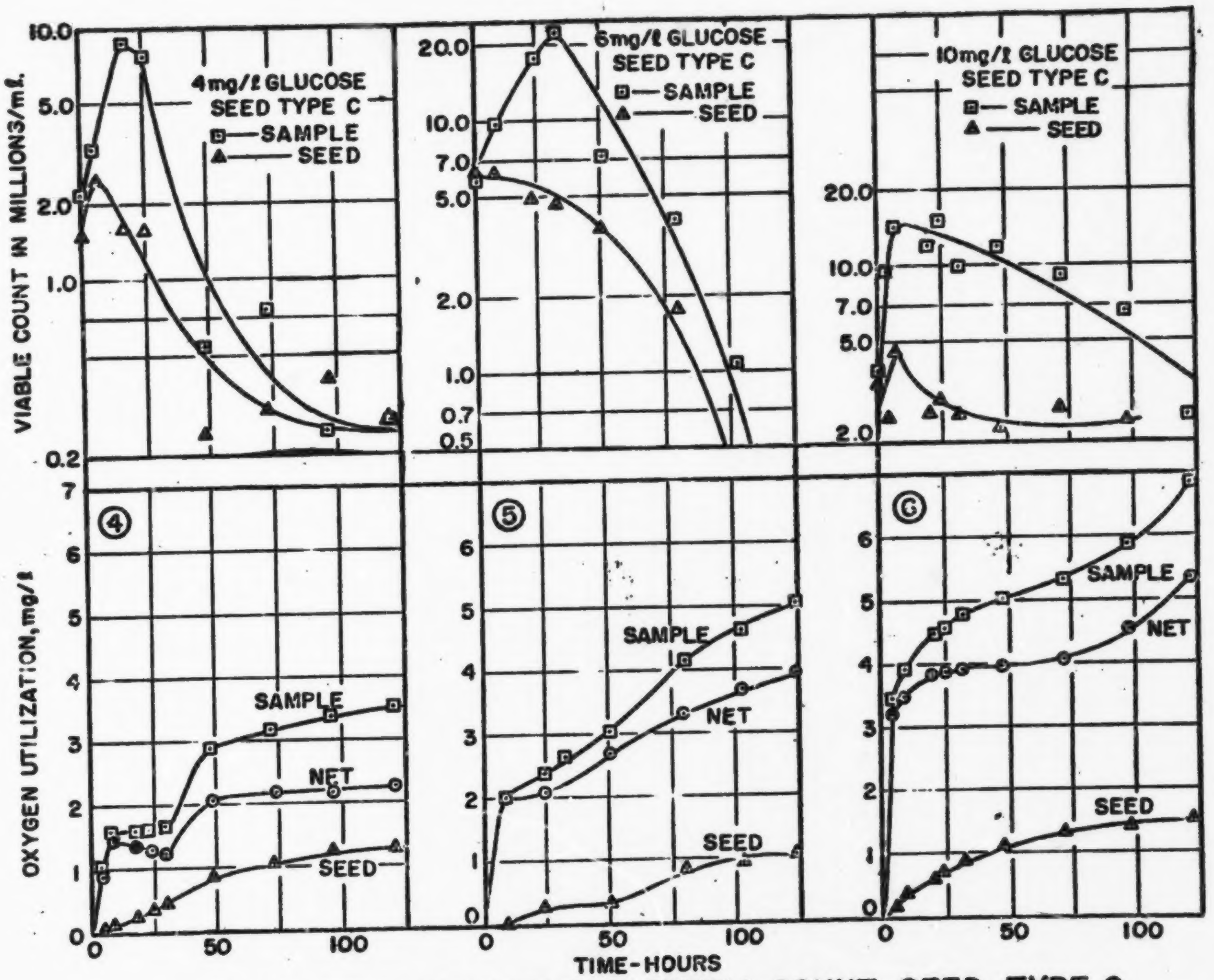

Fig. 4-BOD AND VIABLE BACTERIA COUNT, SEED TYPE C

Experiments are shown for glucose concentrations of 4, 6 and 10 mg/1. The plateau is evidenced in all experiments. It was so pronounced in the experiment shown at the left of the figure that subtraction of the seed yielded an apparent decrease in accumulated oxygen uptake in the sample bottle. In all cases the plateau occurred in the region of maximum population, and the last stage of BOD exertion corresponded to a dieoff in the viable population.

Seed Type D

The results of five experiments using a pure culture of Escherichia coli which was grown, harvested and washed as in seed type C are shown in Figure 5. In this series of experiments two substrate concentrations, 4 and 8 mg/1 glucose, were used, and the initial seed inoculum was maintained above 10^6 bacteria per ml. All experiments yield evidence of a plateau in oxygen uptake. In three experiments multiphase oxygen uptake was also observed for the seed system.

In the figure showing both 4 and 8 mg/1 (experiments 9 and 10) systems, it is seen that the plateau is of longer duration in the system with lower substrate concentration. It is also interesting to note the dependence of oxygen uptake rate during the first stage of oxygen utilization upon substrate concentration. The system shown on the far right yielded a BOD curve somewhat different from the others shown in Figure 5. There would appear to be two plateaus, one occurring in the usual time, and one followed by a decreasing rate portion which then changes to an autocatalytic curve further along the time scale. It should be noted that for most experiments herein

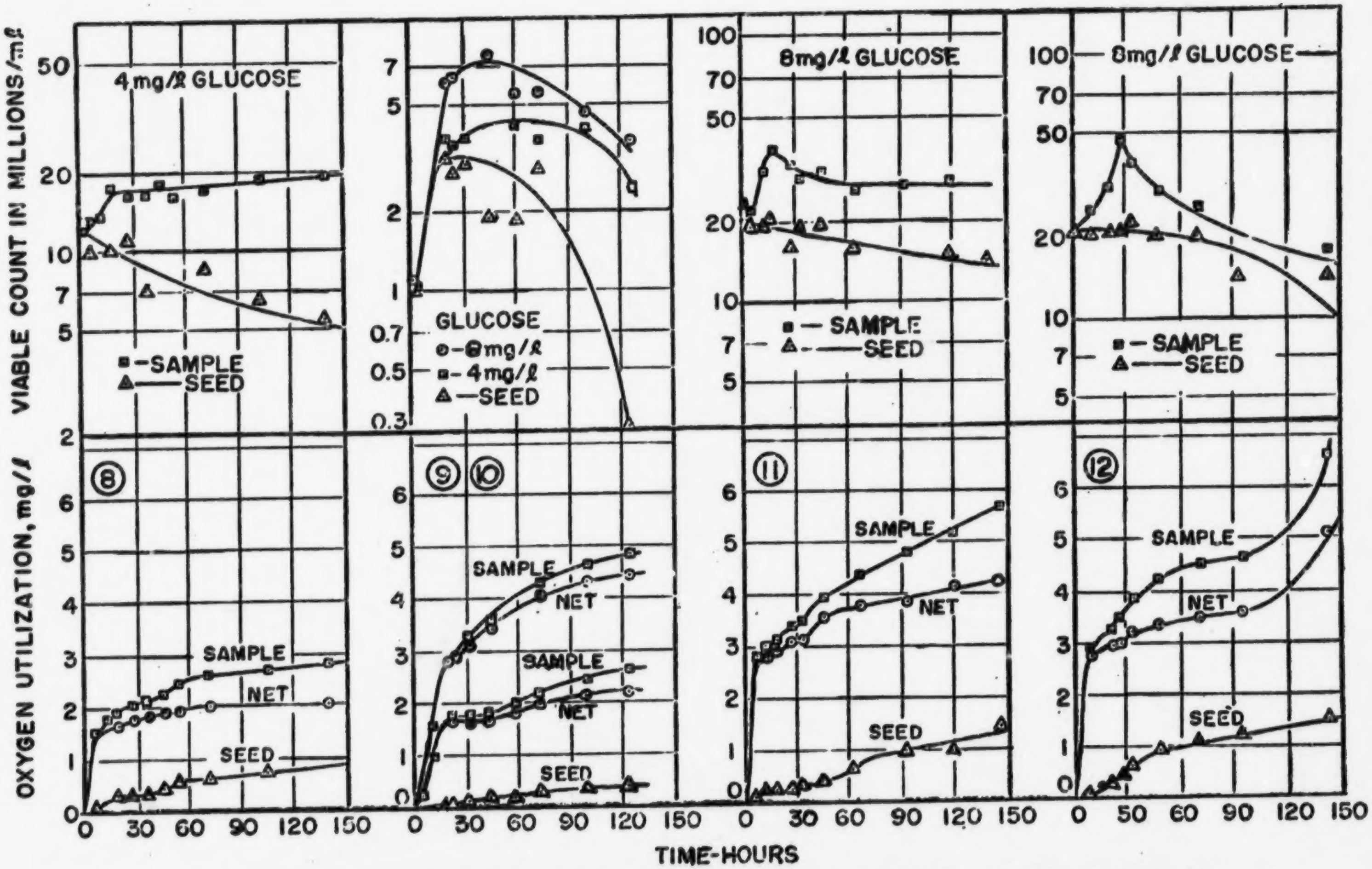

Fig 5 -- BOD AND VIABLE BACTERIA COUNT, SEED TYPE D

presented samples were incubated out to 20 days, but are not shown in order to conserve space. In all such cases the O_2 uptake curves eventually approximated the typical first order decreasing rate curve.

For the experiments shown in Figure 6, the Escherichia coli seed was used to compare results at high and low initial seed populations. In both cases a plateau was observed. It is seen that at the low seed concentration, even though the seed was washed three times, enough substrate was present to provide for approximately six doublings of the population (10^4 to 5×10^5). During this time there was no discernible oxygen uptake as measured by the Winkler method. Oxygen uptake in the seed did not occur until the dieoff period was under way.

The rise in viable population in the seed deserves further discussion. Such a rise is sometimes noted when using low initial seed populations. In experiment 13 there was an increase in viable population of approximately 500,000 cells. Part of the increase might be due to a change in cell volume, but is is difficult to envision how the parent cells could store enough cellular material to provide for more than one doubling. Substrate carryover could account for some of the population increase. These cells were harvested for seed during the log growth phase from a medium in which the initial glucose concentration was 5000 mg/1. A considerable amount of substrate may have been left in the medium at the time of harvesting; however, three centrifugation and washing cycles should have reduced carryover to negligible concentration. It is interesting to calculate the approximate glucose concentration required to produce 500,000 cells. On the average the dry weight of a bacterial cell (coccus or a normal rod) may be taken as

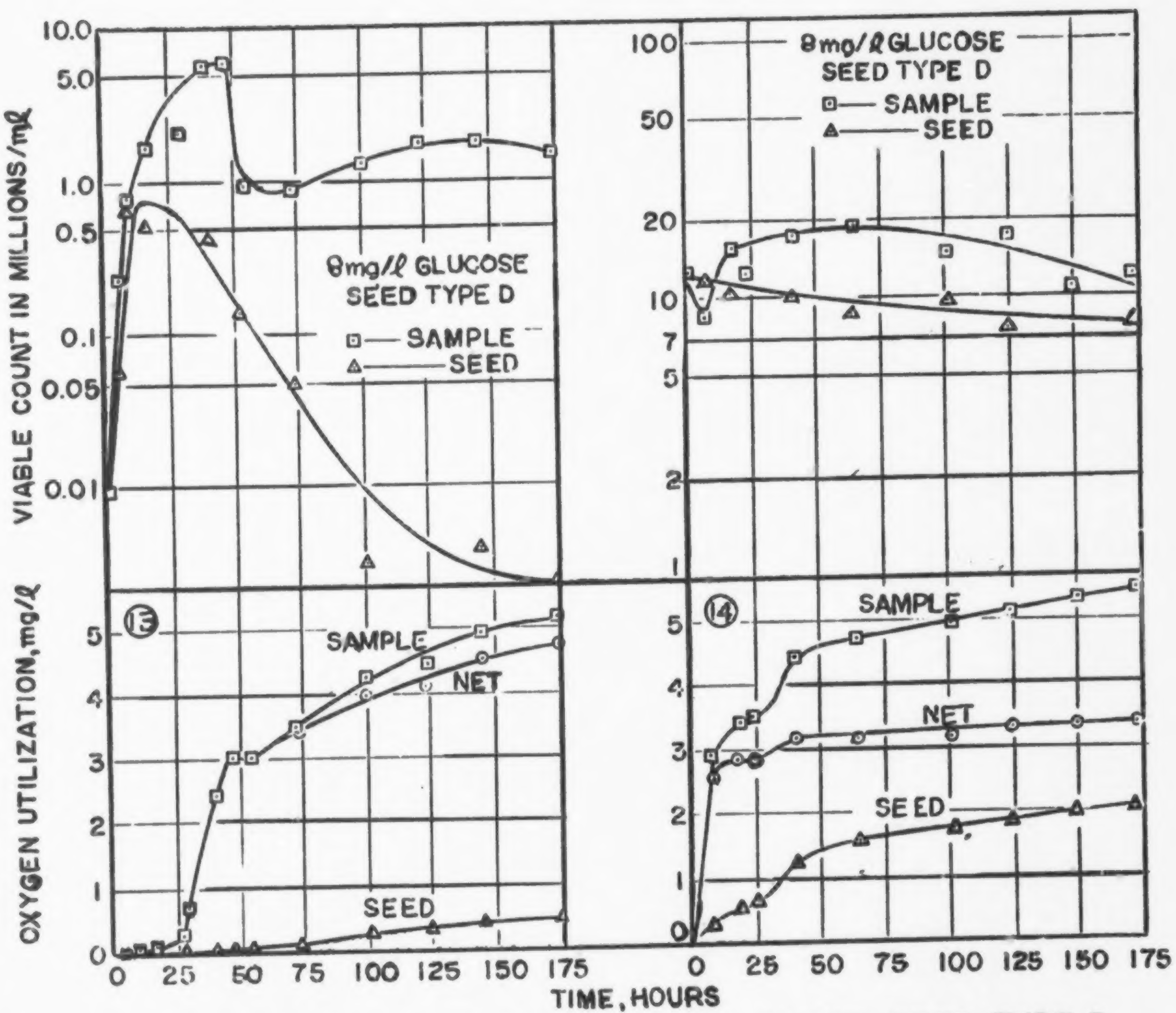

Fig. 6 - BOD AND VIABLE BACTERIA COUNT, SEED TYPE D

2×10^{-10} milligrams, and assuming that 50% of the weight of substrate removed is used for respiration, the following calculation can be made:

$$\left.\begin{array}{l}\text{Substrate channelled into} \\ \text{synethsis to produce} \\ 0.5 \times 10^{6} \text{ cells}\end{array}\right) = \begin{array}{l} 2 \times 10^{-10} \text{ mg/cell} \times 0.5 \times 10^{6} \text{ cells/ml} \\ = 1 \times 10^{-4} \text{ mg/ml} \\ = 0.1 \text{ mg/1} \end{array}$$

Including substrate respired, the food concentration required would be 0.2 mg/1, which is a fairly small concentration. However, it is extremely doubtful that this amount could be carried over. Also, using the above basis of calculation, it is seen that an O_2 uptake of approximately 0.1 falls within an acceptable range of error for D.O. measured by the Winkler method.

Another possible explanation is that the rise in population was due to "declumping." Although there was no discernible clumping or chain formation, and every effort was made to thoroughly disperse the cells, it is quite impossible to say that some cell aggregates did not exist in the initial seed inoculum. In addition it is possible that there were also dead cells in the initial inoculum which after lysis could provide food in addition to carryover substrate. Since all of these possibilities involve extremely low amounts of substrate, their relatively minor effect is not in evidence in the sample systems or when using high initial seed concentrations.

The plateau was also observed for the experiment using a high seed concentration; however, the second stage net oxygen uptake curve was not as pronounced as for the system with lower initial viable count. Oxygen uptake in the seed was very high and was observed to be multiphased, as found for some of the seed curves shown in Figure 5.

The percentage of the theoretical oxygen demand of the substrate exerted up to the plateau varied from 31 to 45%, with an average of 34.2 for the thirteen experiments thus far presented. These values are in reasonable agreement with results shown by Busch(5) and McWhorter and Heukelekian(9).

Experiments on a variety of compounds using young heterogeneous seed (type C) acclimated to the specific compound under study are shown in Figure 7. In order to present all eight experiments in one figure, thus facilitating comparison of the data, it was impossible to show oxygen uptake and population dynamics for the seed. Therefore only net O_2 uptake and viable counts in the sample bottles are shown.

It is seen that there was definite evidence of a plateau in net oxygen uptake for all systems except glycogen and galactose (experiments 2 and 8); however, the total oxygen uptake curve with these experiments (not plotted) showed a slight plateau, which corresponded to one in the seed. Subtraction of the oxygen uptake of the seed yielded a net curve with no plateau in the glycogen system, whereas for galactose the second stage uptake in the seed was more abrupt and upon subtraction yielded a net curve which shows a negative oxygen utilization (see last three points experiment 8). Oxygen uptake of the seed for lactose, ribose, glutamate, sorbitol and melibiose was also diphasic but did not mask the plateau in net oxygen uptake. The total uptake for glycerol gave only a slight indication of a plateau, but the seed uptake broke so sharply that it accentuated the plateau in net oxygen utilization.

Diphasic oxygen uptake in the seed was also noted for some of the glucose experiments previously presented. Although the plateau in BOD expression occurs with and without a similar diphasic occurrence in the seed, it would seem that they are logically related, and since occurrences in the seed are observed to affect the shape of the net curve, it would appear that possible attempts to hasten the course of BOD expression by increasing the seed inoculum should be approached with caution. It would appear at first glance better to use a very low initial seed and accept an autocatalytic curve as the standard BOD curve. However, this would have serious ramifications to the interpretation of the standard five-day BOD test unless the Thomas lag concept can be routinely applied. Also this would not obviate need to consider the plateau; the only advantage that would accrue is that phenomena in the seed would be less felt in net uptake and the shape assumed by the BOD curve would be completely attributable to the substrate or waste sample. The viable count curves for all seed systems were of the same general shpae as the curves shown in Figure 7 for the seed + sample. A slight rise in the seed count of approximately 1/10th of the rise in the sample systems was followed by a dieoff rate not quite as sharp as that exhibited in the sample bottles.

In all experiments shown in Figure 7 exhibiting a plateau it is seen that, in general, the plateau region corresponds to the region of maximum population density. The longer the pause in viable count before the dieoff, the longer is the plateau. The second stage uptake in general corresponds to the region of dieoff in viable count.

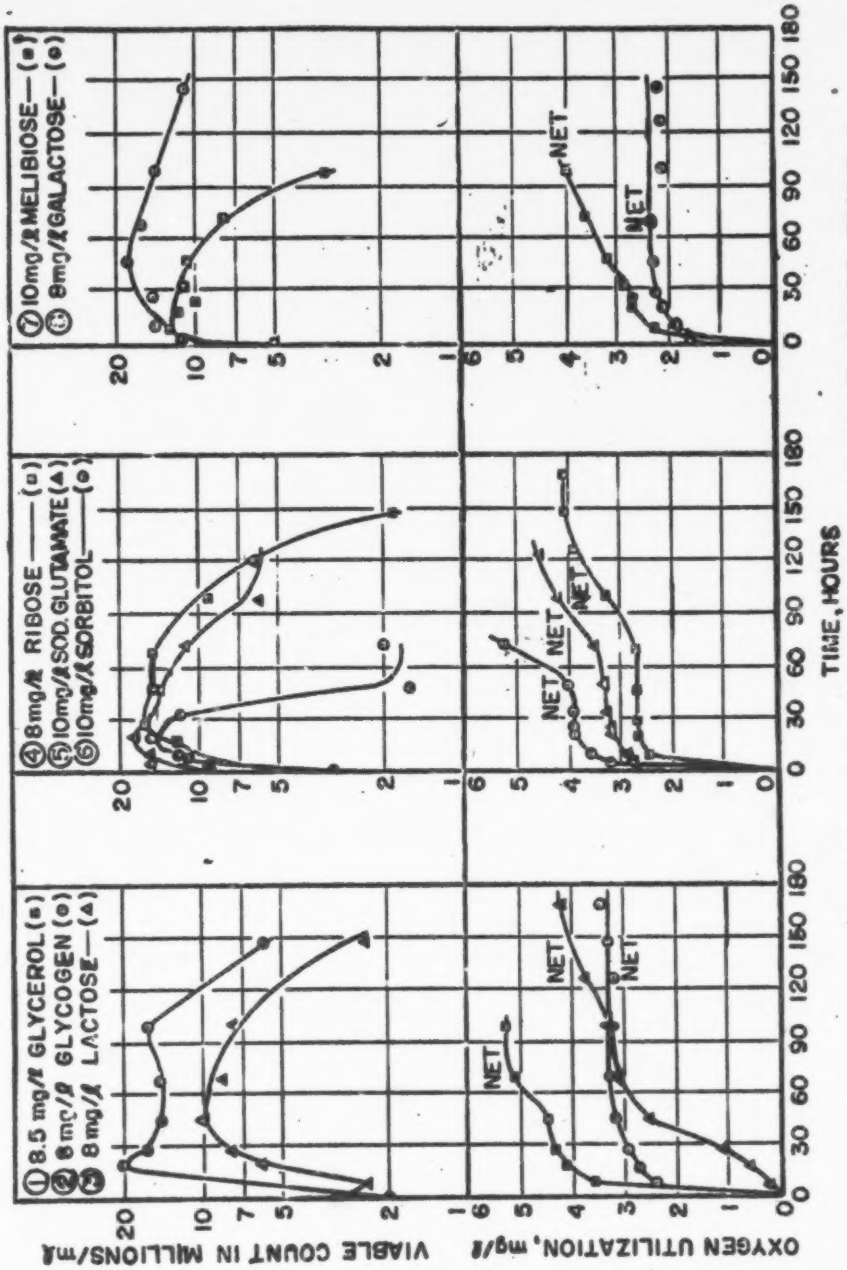

Fig. 7 · BOD AND VIABLE BACTERIA COUNT, SEED TYPE C

Experiments were also run on a variety of multicomponent systems. One such experiment is herein presented.

Glucose + Ribose, Seed Acclimated to Glucose.

Figure 8 shows the course of BOD exertion and viable count for a synthetic waste consisting of 4 mg/1 glucose plus 4 mg/1 ribose. Control systems for glucose and ribose are also shown. The seed was grown on glucose and harvested according to the procedure set forth for seed type C. All systems including the seed exhibited phasic oxygen uptake. Acclimation to ribose took place in less than six hours as seen from the ribose control system, which also shows a plateau at approximately 25 hours. The glucose control and the combined system also exhibited a plateau which occurred at approximately 10 hours. From these results it does not seem that the observed plateau was related to sequential removal of glucose and ribose, since if it were it would not be expected that the combined system would continue to increase after the initial break in the glucose control. Furthermore, a plateau was observed in all systems. It would seem that any diphasic nature of the oxygen uptake curve due to sequential uptake, first on glucose and then on ribose, would have had to occur before the extended plateau which was observed.

DISCUSSION AND CONCLUSIONS

The results herein presented show with reasonable certainty that the plateau in oxygen uptake during exertion of carbonaceous BOD generally exists. It has been shown to occur using sewage seed, laboratory pilot plant effluent, washed heterogeneous bacterial suspensions,

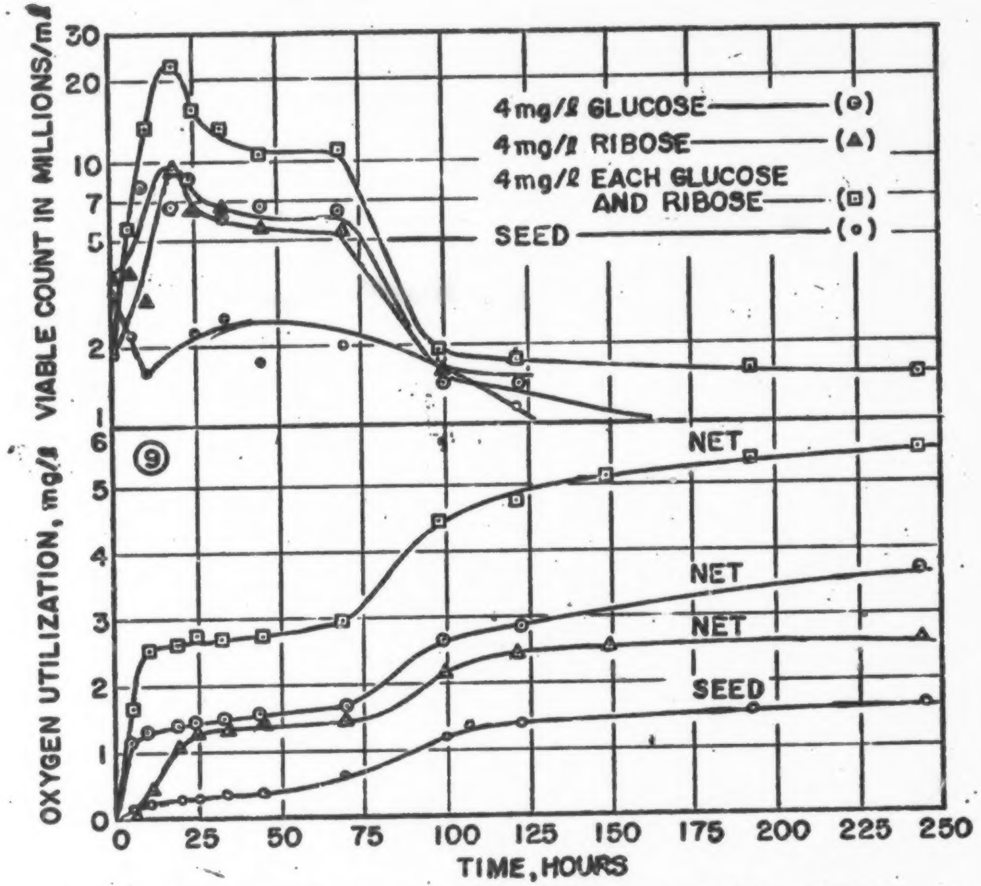

Fig. 8 – BOD AND VIABLE BACTERIA COUNT, SEED TYPE C ACCLIMATED TO GLUCOSE.

and a pure culture of Escherichia coli. It has been found to exist using various concentrations of initial seeding material and using various types and concentrations of carbon sources. In addition to the work herein presented it has been found to exist in other experiments in our laboratory, and it has been shown by the work of Busch,(5) McWhorter and Heukelekian,(9) and Wilson and Harrison.(10) While all of these experiments attest to the generality of its occurrence, and while it may be stated that the plateau usually occurs at a point between 30 and 40% of the theoretical oxygen demand, little can be stated concerning its severity and duration, and thus its effect upon predictive evaluation of the kinetics of BOD exertion. Therefore it would seem that the plateau value is of little aid in attempting to formulate a mathematical expression which describes the entire course of BOD exertion. It was seen in these studies that the plateau in some cases was extremely pronounced and in other cases represented only a slight pause or flattening of the BOD curve.

Under "normal" conditions of seed and substrate concentrations the first stage in oxygen uptake was found in these studies to correspond to a rising viable population curve, the plateau to the region of maximum cell density and the second phase of O_2 uptake to a decrease or dieoff portion of the viable count curve. Although this correlation was generally found, this should not be taken to indicate that diphasic oxygen uptake in the BOD bottle must always be accompanied by rapid growth and dieoff of the viable population. When using low seed concentrations this correlation would seem reasonable; however, the plateau was also observed using abnormally high concentrations of initial seed, which militated against multifold increases in viable population. The plateau also occurred in these high seed concentrations for cases where a multifold dieoff was not observed. However, the correlation existed in enough cases to be cited as a general phenomenon. However valid this correlation may be in the general case, it does not explain the reason for a pause or plateau in oxygen uptake.

Based upon the results herein presented and others we have obtained in high substrate concentration systems, it is possible to predict various mechanisms of causation. The following possibilities seem prominent:

(1) In a heterogeneous cell system, after reaching the stage of maximum growth (cell production or combination of cell production and storage), the predominating cells begin to die or otherwise lose their turgor and release cellular components which may become food for other species of cells. Since these were not the predominating species, time is required before they can grow to such numbers that their growth is expressed in oxygen uptake. This might lead to two autocatalytic curves; the end of the first phase and the beginning of the second form the plateau.

(2) The phenomenon cited above occurs without the aftergrowth of a second species, that is, there is no change in predominance, but because of the different structural configuration of the original substrate and the released cellular components an acclimation period is required to synthesize the new enzymes needed to metabolize the new, and now exogenous, substrates. If enough cellular components are released, an aftergrowth may occur; on the other hand, the released components may not be of sufficient quantity to support much new growth,

and the dieoff in total population continues. It is also possible that the released components may provide enough energy to maintain the viable integrity of the cells, and cell dieoff proceeds more slowly.

Theories (1) and (2) differ only in that the secondary stage of O_2 uptake is caused by a new predominance in the population in theory (1), and by the same population with a required acclimation period in theory (2). The fact that the cells themselves may become food for protozoa would seem to form an incidental part of theory (1). The fact that the plateau is short in some cases and long in others can arise by a combination of (a) time for acclimation, and (b) time to gain enough population to be expressed in oxygen uptake.

(3) Cells do not die in the sense that they no longer can respire, but they may lose their ability to replicate. An acclimation period is needed before cellular components formerly synthesized in replicating processes can be broken down to supply energy. Another possibility within the scope of theory (3) is that an acclimation period is needed before the cells can use certain "storage products" for energy. In this case the length of the plateau, and indeed its existence in such systems, would depend upon the nature of the storage products and the induction time, if required, for producing the necessary catabolic enzymes. Reactions of this type might provide an explanation for the delayed onset of endogenous respiration suggested as a possible cause for the plateau by Wilson and Harrison.(10)

(4) During rapid metabolism of some compounds the cells release intermediates into the medium rather than channeling all such compounds into storage products. In such cases time may be required

for induction of enzymes to initiate metabolism of the new exogenous compounds. Time for the induction of transport catalysts may also be required to bring these new components to existing enzyme systems within the cell. Since there is no longer enough substrate to support continued growth, the cells begin to die off.

It may be discerned that although all four theories differ in essential components, there is one central likeness. The plateau is caused by a change from metabolism of the original exogenous carbon source(s) to metabolism of new carbon source(s) produced by the cells. These may be excreted after maximum population is reached, or during rapid metabolism, or may remain in the cells, but the nature of the carbon source the cells are required to metabolize changes. This may or may not lead to changes in species predominance. In any event when the change in carbon source is abrupt and an induction period is required, a plateau can occur.

SUMMARY AND CONCLUSIONS

1. The occurrence of a plateau in BOD exertion according to the results herein presented would appear to be independent of the type of seed employed.

2. In general under "normal" seeding conditions and substrate concentrations in the BOD bottle, the first phase of oxygen uptake corresponds to a period of rapid cellular multiplication, the plateau occurrence to the maximum range of population density, and the second stage of oxygen uptake to a decrease in the viable population.

3. The duration or severity of the plateau corresponds approximately to the length of the stationary phase in the region of maximum population.

4. Four related theories explaining the existence of the plateau were discussed. These theories are similar in the sense that the plateau is caused by a change from metabolism of the original exogenous carbon source to the metabolism of the new carbon sources produced by the cells. The plateau comes about because of the need of an induction period before the new energy source(s) can be metabolized to such an extent that this metabolism is expressed in oxygen uptake.

Acknowledgement

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PAPER 7

STUDIES ON THE OCCURRENCE OF THE PLATEAU IN BOD EXERTION. III. SIMPLE AND COMPLEX WASTES WITH VARIOUS SEED AND SUBSTRATE CONCENTRATIONS

By

R. H. Follett and A. F. Gaudy, Jr.

This paper was presented at the 14th Oklahoma Industrial Wastes Conference, November, 1963, and portions of the data were published in Paper 8. (See note, Paper 4.)

STUDIES ON THE OCCURRENCE OF THE PLATEAU IN BOD EXERTION. III. SIMPLE AND COMPLEX WASTES WITH VARIOUS SEED AND SUBSTRATE CONCENTRATIONS[1]

By

R. H. Follett[2] and A. F. Gaudy, Jr.

The well established first order decreasing rate curve for BOD exertion described by the equation

$$y_t = L(1 - 10^{-kt})$$

has become subject to considerable doubt due to observation of phasic kinetics and, more recently, of a pause or plateau between phases of carbonaceous BOD expression. It would seem quite natural to ascribe such kinetic behavior in a gross sense to the particular growth patterns of the biological population exerting the BOD. These growth patterns in turn might be controlled in large measure by the chemical composition of the waste under study.

Hoover, Jasewicz and Porges observed diphasic expression of BOD using milk wastes(1). The first phase was attributed to the rapid growth of cells with assimilation of available nutrients into the cells, and the second phase to a slower endogenous respiration. Buswell, Mueller and Van Meter also described the BOD exertion curve as diphasic with the first phase attributable to cell multiplication and the second phase associated with a resting or dying culture(2). Zehnpfennig and Nichols felt that the first stage of oxygen uptake is controlled by substrate concentration, and the second phase is determined by the number of bacteria and the reproduction rate of these bacteria rather than substrate concentration(3). Busch, et al. have observed diphasic carbonaceous BOD expression in which the two phases are separated by a plateau(4)(5)(6). According to Busch and his co-workers, the first phase represents the conversion of available substrate to cell material and storage products. The second stage consists of a combination of endogenous respiration by the

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[1] Presented at 14th Oklahoma Industrial Wastes Conference, November 20-21, 1963.

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cells and the metabolism of the cells by predators. It was felt that a large number of protozoa in the seed inoculum masked the plateau. In these investigations, data on population dynamics were not obtained. Tidwell and Sorrells have presented data from which they conclude that BOD of a given waste varies not only with the amount of organic matter present, but also with the number of organisms capable of oxidizing that organic matter, and not necessarily with the total number of organisms present in the system(7). Their BOD data also indicate a plateau in oxygen uptake. Some of the work cited above is discussed in more detail in previous reports(8)(9). The major point to be reemphasized is that there is a considerable amount of published work which indicates that diphasic oxygen uptake, and often a plateau between phases of uptake, exists in many cases.

If diphasic BOD expression truly does exist in the majority of cases, the prevalently used first order decreasing rate concept should be subject to considerable review, since the BOD test is by far the most widely used parameter for measuring the pollutional potential of a waste. In our laboratories oxygen uptake and population dynamics have been studied in attempts to gain a greater insight into the kinetics and mechanisms of substrate utilization in dilute systems such as exist in the BOD bottle and in the receiving stream. We have obtained results which showed that the occurrence of a plateau in BOD exertion was independent of the four types of seed thus far employed (sewage seed, acclimated seed, washed acclimated seed, and a pure culture of *Escherichia coli*). In general, under normal seeding conditions and substrate concentrations the first phase of oxygen uptake has been found to correspond to a period of rapid bacterial multiplication. The plateau corresponds to the period of maximum cell density, while the second stage of oxygen uptake corresponds to a decrease in the viable population. Based upon these results, four related theories of causation for the existence of the plateau and diphasic oxygen uptake have been presented(8)(9). Work presently under way is designed to test these theories and to extend the work to both high and low energy systems.

From an engineering viewpoint, determination of factors which affect the occurrence of the plateau is as immediately significant as determination of mechanisms of causation. It was therefore felt that the line of investigation previously reported should be extended to include determination of the effects of substrate concentration and the size of the initial seed inoculum. It was also felt that the work should be extended to include studies on complex media and whole wastes since most of the work showing the plateau has been accomplished using simple substrates.

MATERIALS AND METHODS

The methodology and techniques used throughout these investigations were essentially the same as those previously reported(8)(9). Dissolved oxygen was measured using the Alsterberg modification of the Winkler method. At each sampling period, before adding Winkler reagents, 1 ml was withdrawn from the BOD bottle, appropriately diluted, and used to determine the viable bacteria count, using the spot plate method(10).

Three types of seeding material were used in these studies. Seed type A consisted of effluent from the primary clarifier of the municipal sewage treatment plant at Stillwater, Oklahoma. Seed type B consisted of effluent from a batch activated sludge unit fed on the carbon source to be used in the BOD experiments. Seed type C' was the same as seed type B except that the cells were harvested by centrifugation and washed once with 0.05 molar phosphate buffer, pH 7.0. The designation C' is herein used in order to distinguish this washed seed type from "young cell" washed seed type C used in previous reports(8)(9). The laboratory activated sludge units from which seed types B and C' were obtained were started with an initial inoculum of seed type A. The complex media used in these studies consisted of nutrient broth, sterile sewage, neutral sulfite semichemical pulp liquor, Kraft mill digester liquor, and Kraft mill effluent. The only pure compound employed as substrate was glucose.

RESULTS

EFFECT OF SUBSTRATE CONCENTRATION

Figure 1 shows oxygen uptake using glucose concentrations of 2, 5 and 8 mg/l. Diphasic kinetics are exhibited in all systems including the seed. Although there is some doubt as to the location of the plateau or change in kinetics for the 5 mg/l concentration (see O_2 value at 84 hours), and for the 2 mg/l concentration (see

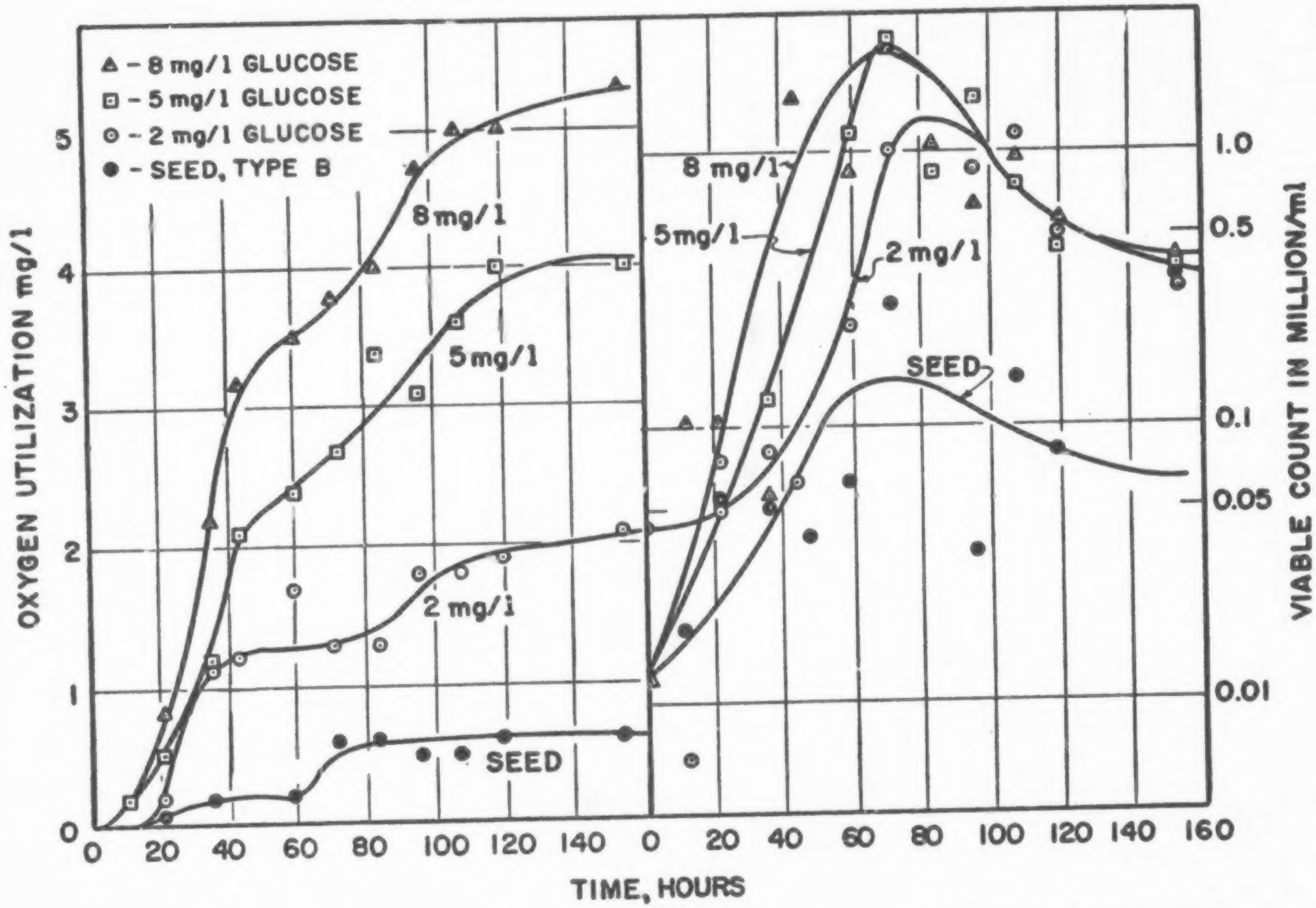

FIGURE 1. Oxygen Uptake and Viable Bacteria Count in the BOD Bottle for Various Concentrations of Glucose using Acclimated Seed.

O_2 value at 60 hours), there is little doubt that two phases of oxygen uptake existed. As in the previous studies, the second phase was associated with a dieoff of the bacterial population. This experiment was repeated using a lower initial cell concentration, and the plateau was again evidenced; it corresponded approximately to the range of maximum population density.

Results of experiments in which 12 mg/l glucose were used are shown in Figure 2. In both experiments phasic kinetics were exhibited even though the initial seed concentrations for these experiments were quite different. The discontinuity in oxygen uptake was more pronounced in the experiment (B) with lower initial seed concentration. It is also interesting to note that the viable population in experiment B grew to significantly higher numbers than in experiment A. The viable count data indicate once again that the second stage of oxygen uptake occurs after attainment of maximum viable population. Because of the fairly high substrate concentration employed, there may be some question concerning a possible nitrogen deficiency. Although a substrate concentration of 12 mg/l probably approaches this situation under standard BOD testing conditions (1.7 mg/l NH_4Cl), a serious nitrogen limitation does not seem probable based upon calculations and considerations presented in the discussion.

EFFECT OF SEED CONCENTRATION

Results are presented in Figures 3, 4 and 5 for systems in which initial seed concentration (type C') was varied from 1×10^3 to 1×10^7 cells per ml. In all experiments the substrate consisted of glucose at 8 mg/l. Although the seed for each experiment was obtained from the same laboratory batch activated sludge unit, the oxygen uptake and viable population patterns varied drastically. This can be seen by comparing the seed curves in Figures 3 and 4; in Figure 3 the initial seed inoculum was only 10^3 cells per ml. The population increased to approximately 10^5 cells per ml (or an increase of approximately 100,000). Oxygen uptake of the seed was relatively high. In Figure 4 the initial seed concentration was approximately 10^5 cells per ml and the highest population attained was approximately 5×10^5 (or an increase of approximately 400,000) and oxygen uptake was much lower than that observed in Figure 3. Although there are differences in oxygen uptake and growth curves for these

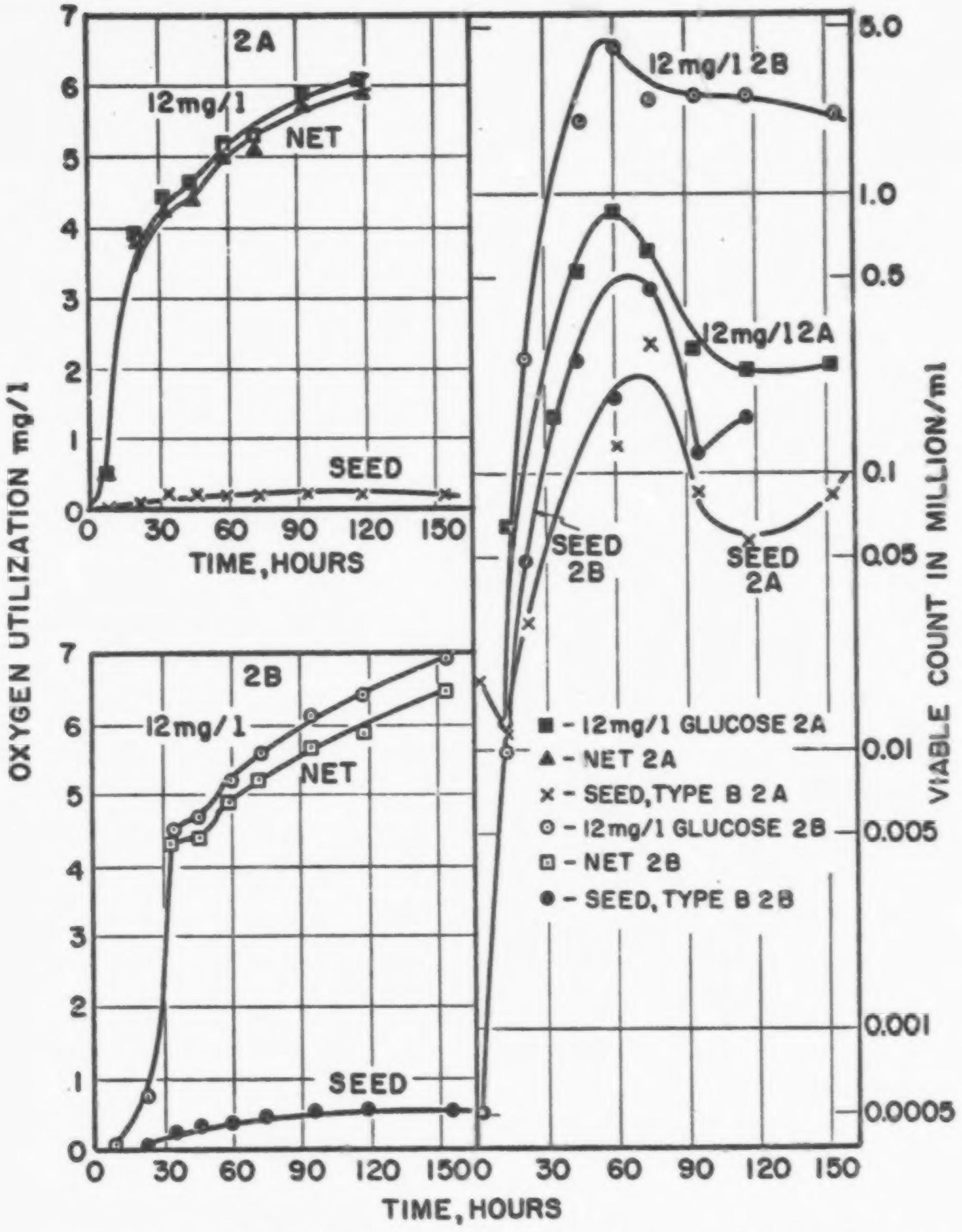

FIGURE 2. The Effect of Initial Seed Concentration on Oxygen Uptake and Viable Bacteria Count using Acclimated Seed and 12 mg/1 Glucose.

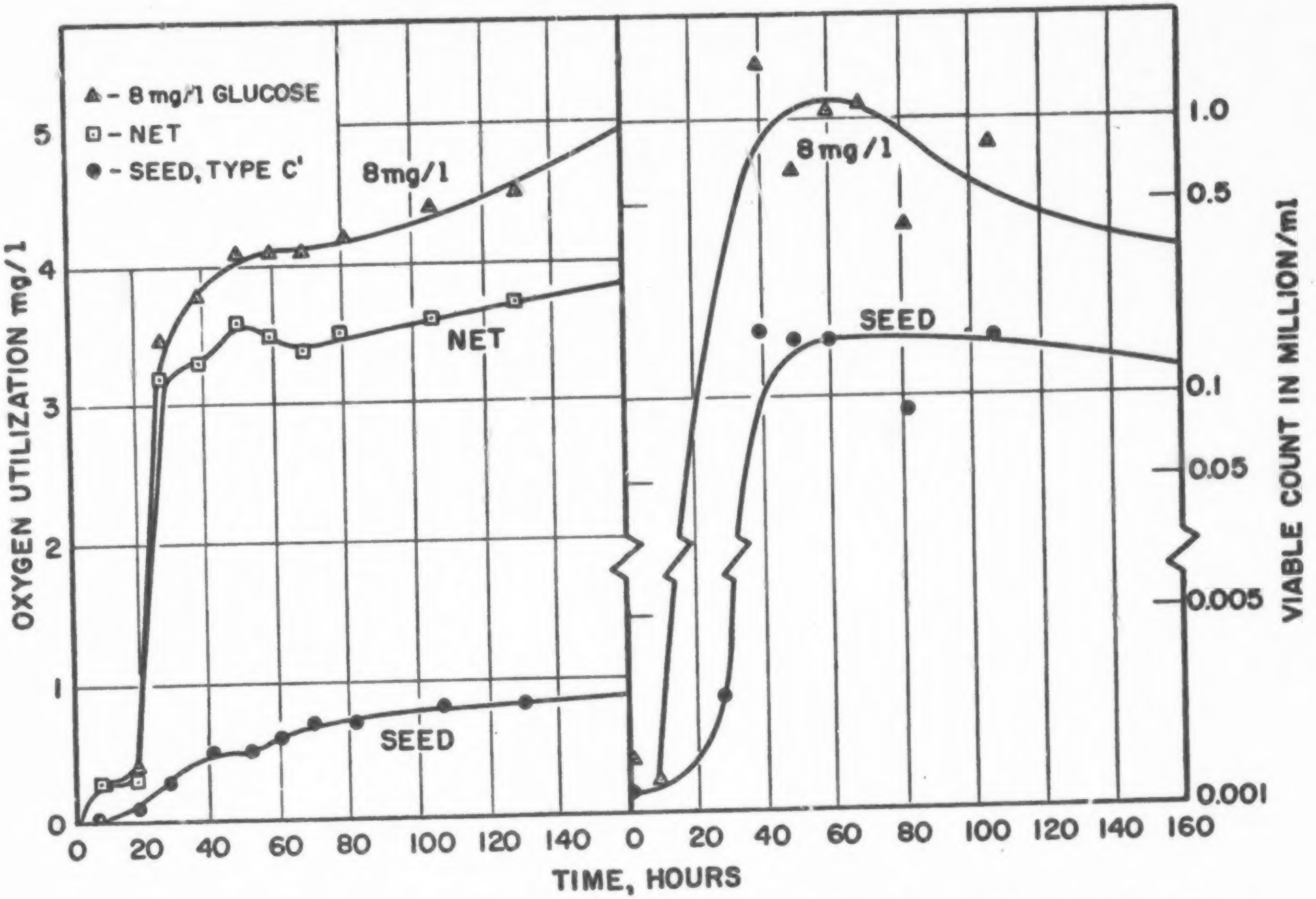

FIGURE 3. Oxygen Uptake and Viable Bacteria Count in the BOD Bottle using 8 mg/l Glucose and a Low Initial Concentration of Acclimated Cells.

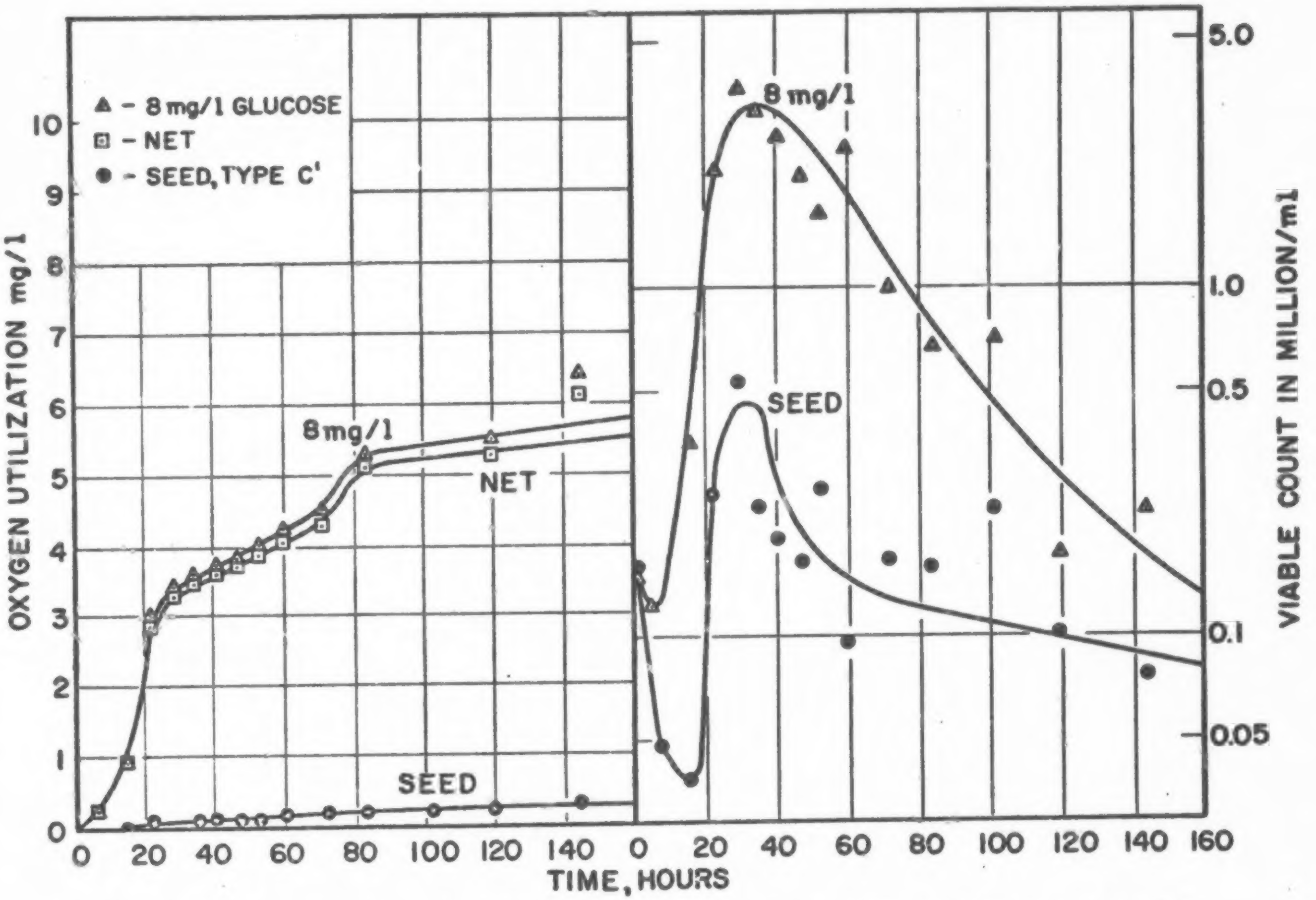

FIGURE 4. Oxygen Uptake and Viable Bacteria Count in the BOD Bottle using 8 mg/l Glucose and a High Initial Concentration of Acclimated Cells.

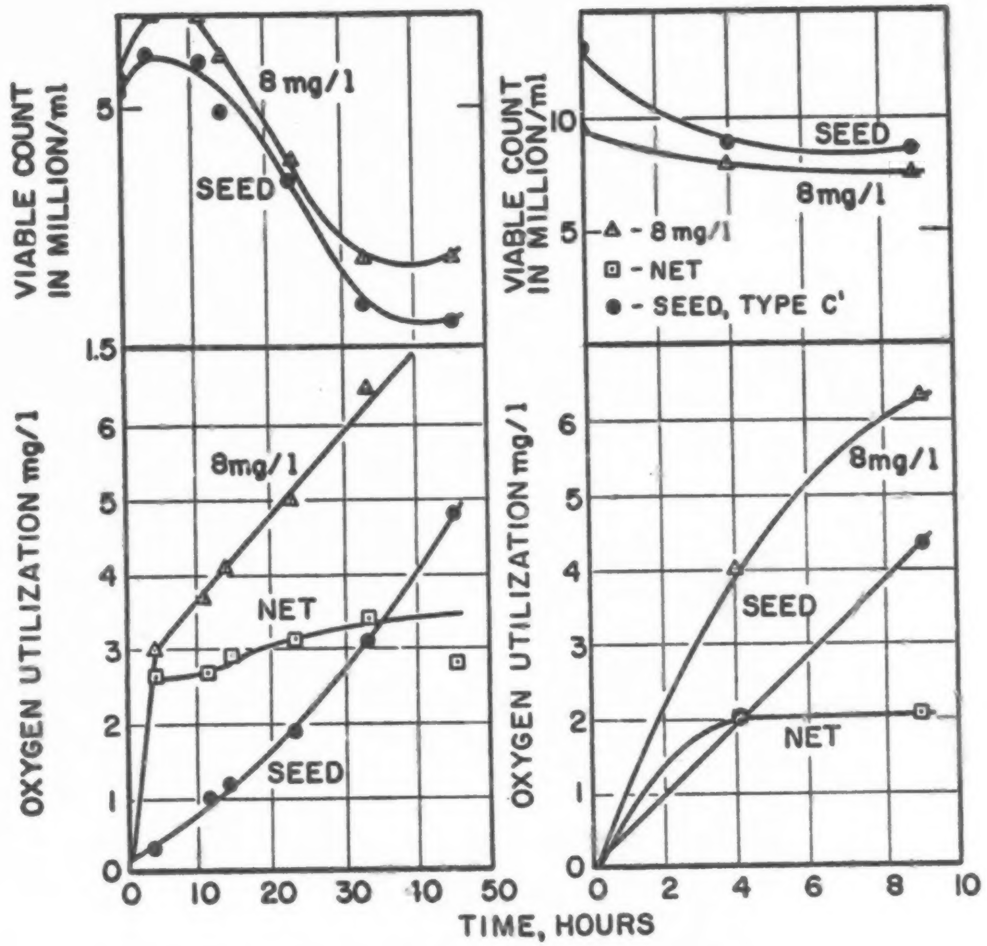

FIGURE 5. Oxygen Uptake and Viable Bacteria Count in the BOD Bottle using 8 mg/l Glucose and Abnormally High Initial Concentrations of Acclimated Cells.

experiments, the general pattern remains as before; the BOD curves are discontinuous, i.e., they indicate either plateaus or changes in kinetics, and the kinetic change is associated with attainment of maximum viable population.

Figure 5 shows the results of two experiments in which extremely high initial cell inocula were used. At an initial population of 5×10^6 cells per ml there is definite indication of phasic kinetics, and this is associated with attainment of maximum viable count. When a higher initial population (10^7 cells per ml) was used in systems containing 8 mg/l glucose, the data show a decrease in population. It is emphasized, however, that very few sampling points were obtained in this experiment. It is interesting to note that the net O_2 uptake in this system was lower than in any of the others.

All results thus far presented indicate that phasic oxygen uptake kinetics hold for systems employing a simple carbon source such as glucose, with the exception of the experiment in which an initial inoculum of 10^7 cells per ml was used. The discontinuity in kinetics always occurs in the range of maximum population density. The discontinuity generally occurs as a plateau, and the second phase of oxygen uptake corresponds to a decreasing viable count. For all experiments accomplished using glucose as substrate in this study, calculations were made pertaining to the percentage of theoretical oxygen uptake at which the plateau occurred. On the average, the plateau occurred at 37% of the theoretical oxygen demand of the substrate. This is in general agreement with values previously reported from our laboratory and others(4)(8)(11).

EFFECT OF COMPLEX SUBSTRATES

Previous results obtained in our laboratory using a wide variety of simple carbon sources and results obtained by others indicate that the occurrence of a plateau or of phasic kinetics during BOD exertion is independent of the type of substrate (4)(5)(8)(9)(12). However, natural waste waters contain many more types of carbon source than have been studied with regard to the plateau. In addition few, if any, wastes are composed predominantly of one carbon source. It was therefore felt that studies using a complex medium and various waste waters would be of considerable value. Figure 6 shows results of an

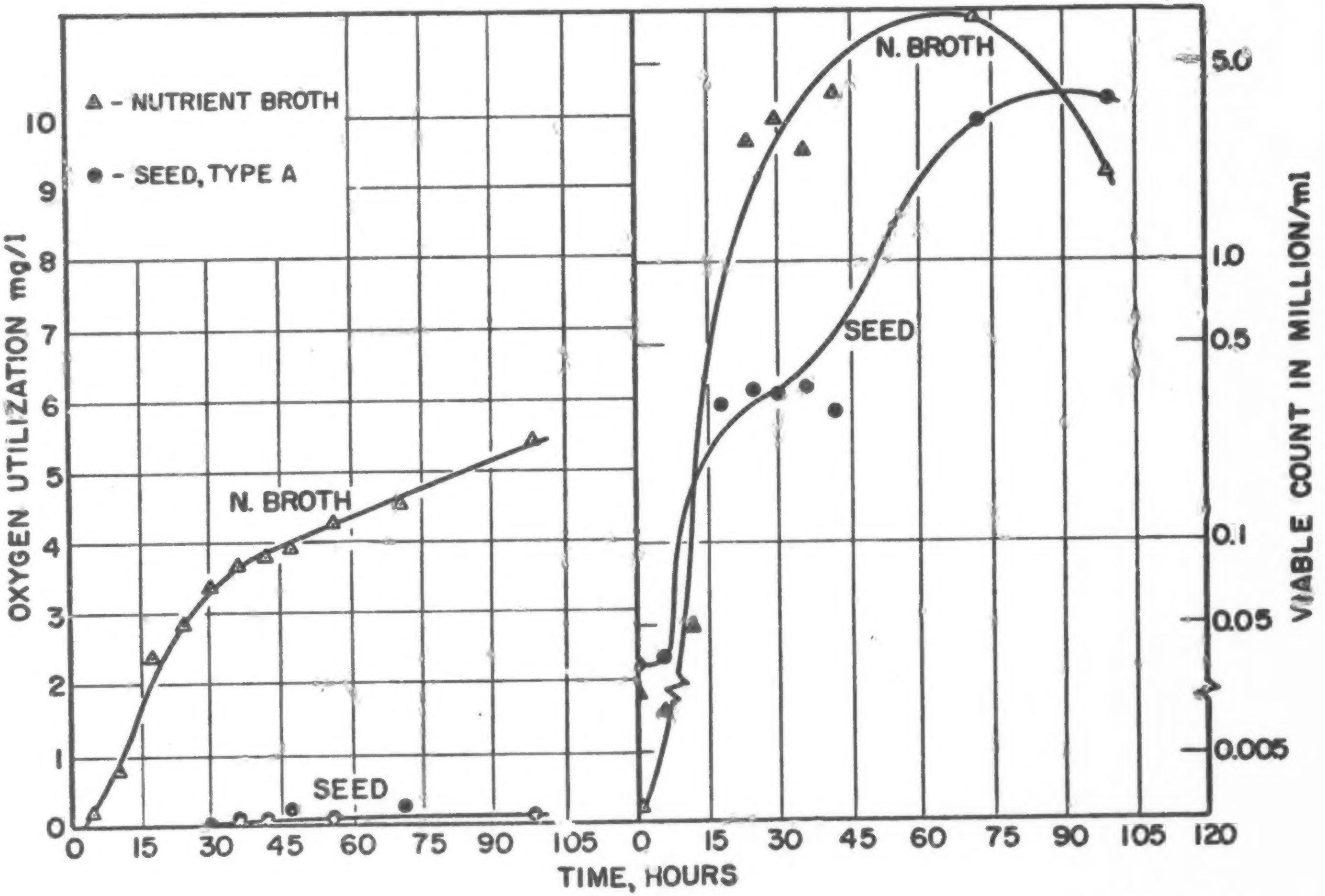

FIGURE 6. Oxygen Uptake and Viable Bacteria Count in the BOD Bottle using Nutrient Broth and a Sewage Seed.

experiment in which dilute nutrient broth (Difco) was used (1.875 ml/l of dilution water)[1]. This medium contains Bacto-beef extract and Bacto-peptone. Sewage seed (type A) was employed. Two distinct phases of oxygen uptake were observed, but there was little evidence that these were separated by a plateau. From the viable population data obtained it would appear that there is a rather broad range of maximum population density. This experiment was repeated using a higher dilution of nutrient broth (0.625 ml/l dilution water)[2]. The same general trends were observed; there was only slight evidence of a plateau.

Sewage obtained from the primary settling tank effluent of the Stillwater municipal treatment plant was employed as substrate at a dilution of 40 ml/l of dilution water for the experimental results shown in Figure 7. The portion employed as substrate was sterilized before use, while an unsterilized portion (0.25 ml/l dilution water) of the same sewage sample was used as seed material. These results demonstrate the full gamut of BOD kinetics typified by dual phases separated by a plateau. It is clearly seen that the plateau corresponds to the range of maximum population density, and that the second stage is associated with a dieoff in viable population.

Digester liquor from a neutral sulfite semi-chemical wood pulp operation was also used as substrate at a concentration of 0.1875 ml/l[3] (see Figure 8); the seeding material consisted of washed acclimated cells (seed type C'). The results yielded evidence of a slight plateau and the second phase of oxygen uptake was associated with a decrease in viable population and a slight regrowth. A second experiment verified the trends herein shown.

The results of experiments shown in Figures 9 and 10 were obtained using washed seed acclimated to Kraft mill (digester) black liquor. Figure 9 shows results using the digester liquor as substrate, while for the results given in Figure 10 total Kraft mill effluent, i.e., Kraft mill waste after being subjected to the recovery process inherent in the Kraft operation, was employed. Identical seeding material was used in both cases. It is seen that a plateau in O_2 uptake occurred for both wastes. While the plateau

1. 15 ml standard nutrient broth in 8 liters BOD dilution water.
2. 5 ml standard nutrient broth in 8 liters BOD dilution water.
3. 1.5 ml digester liquor in 8 liters BOD dilution water.

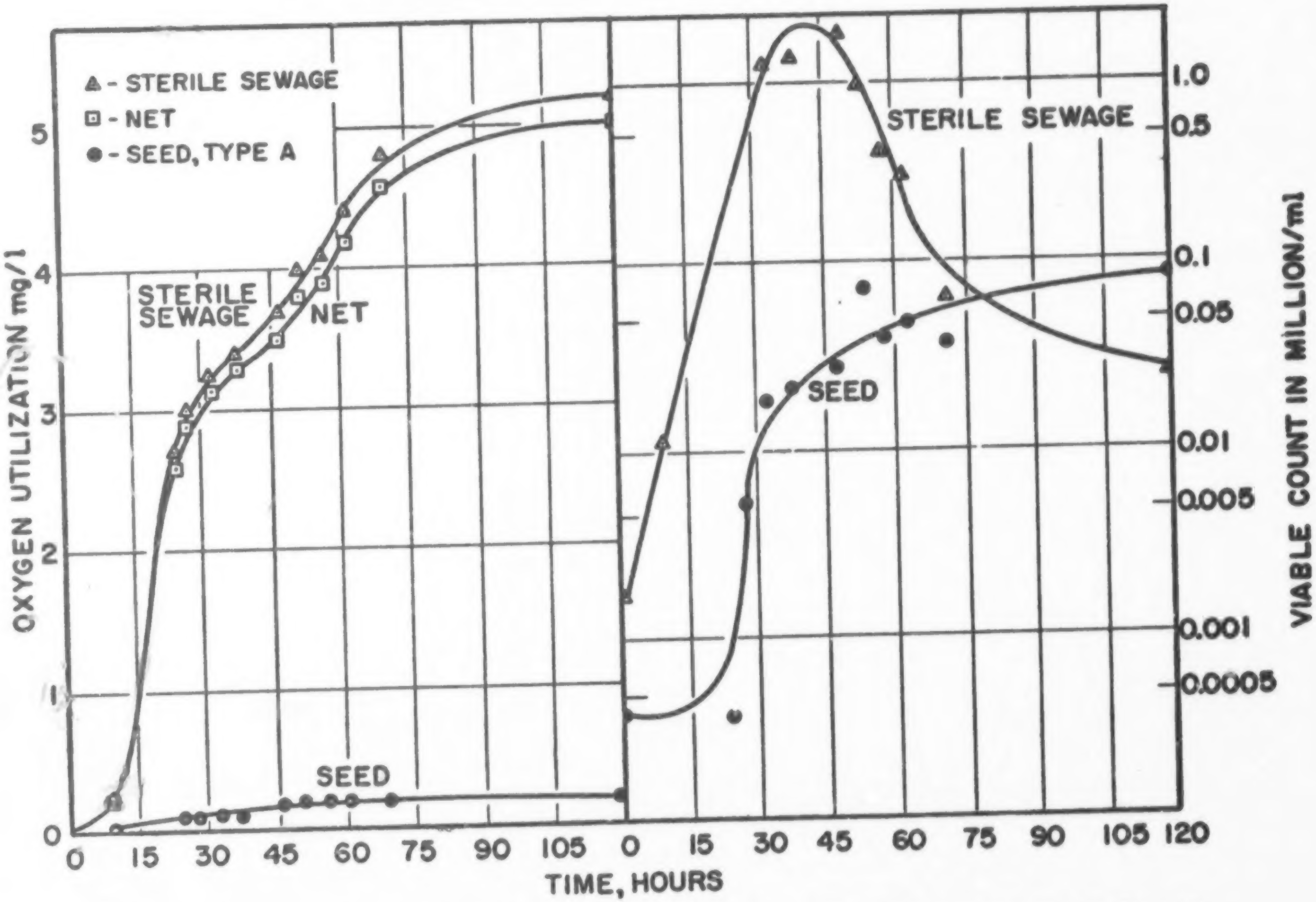

FIGURE 7. Oxygen Uptake and Viable Bacteria Count in the BOD Bottle using Sterile Sewage and a Sewage Seed.

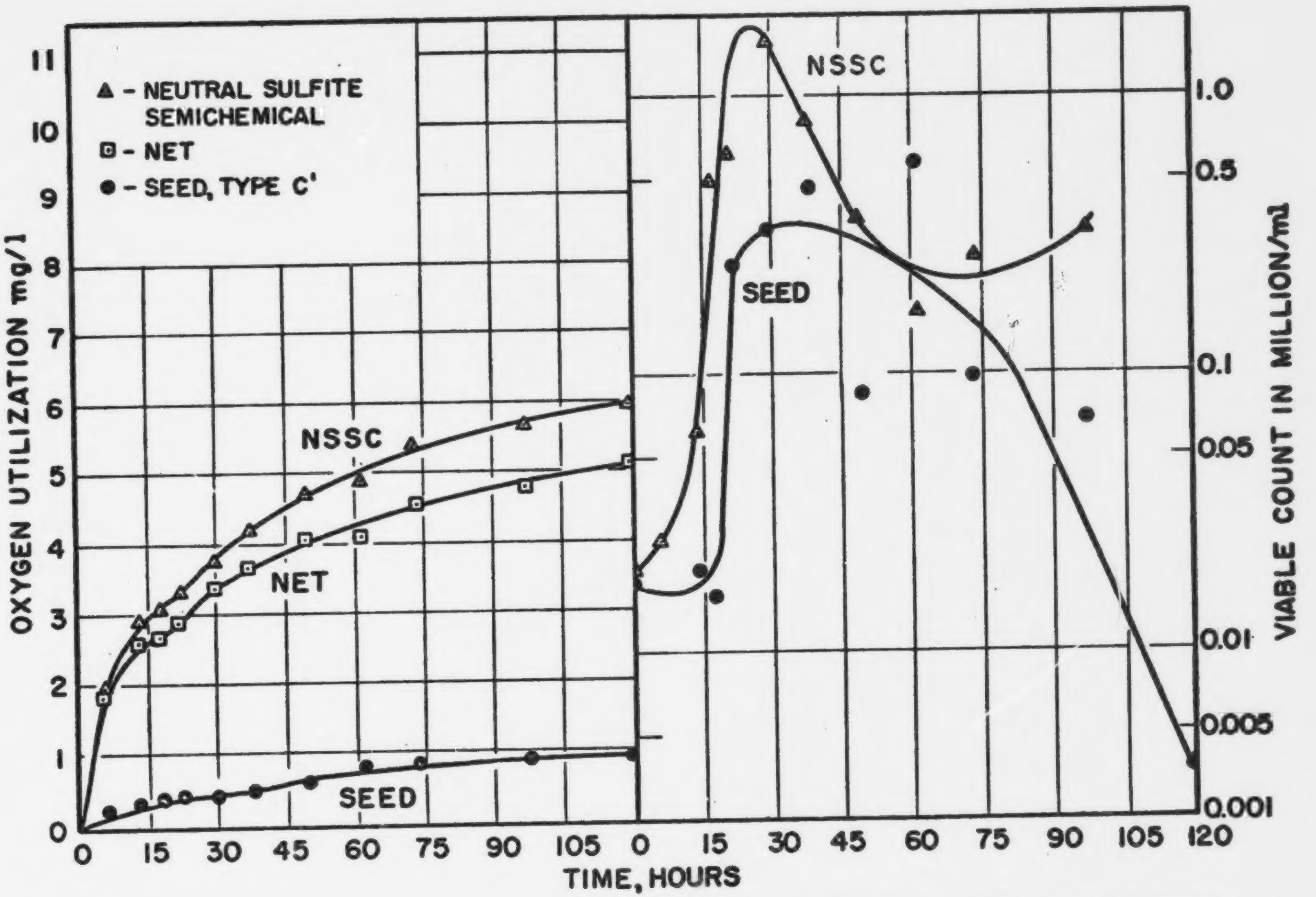

FIGURE 8. Oxygen Uptake and Viable Bacteria Count in the BOD Bottle using Neutral Sulfite Semi-chemical Waste and a Washed Acclimated Seed.

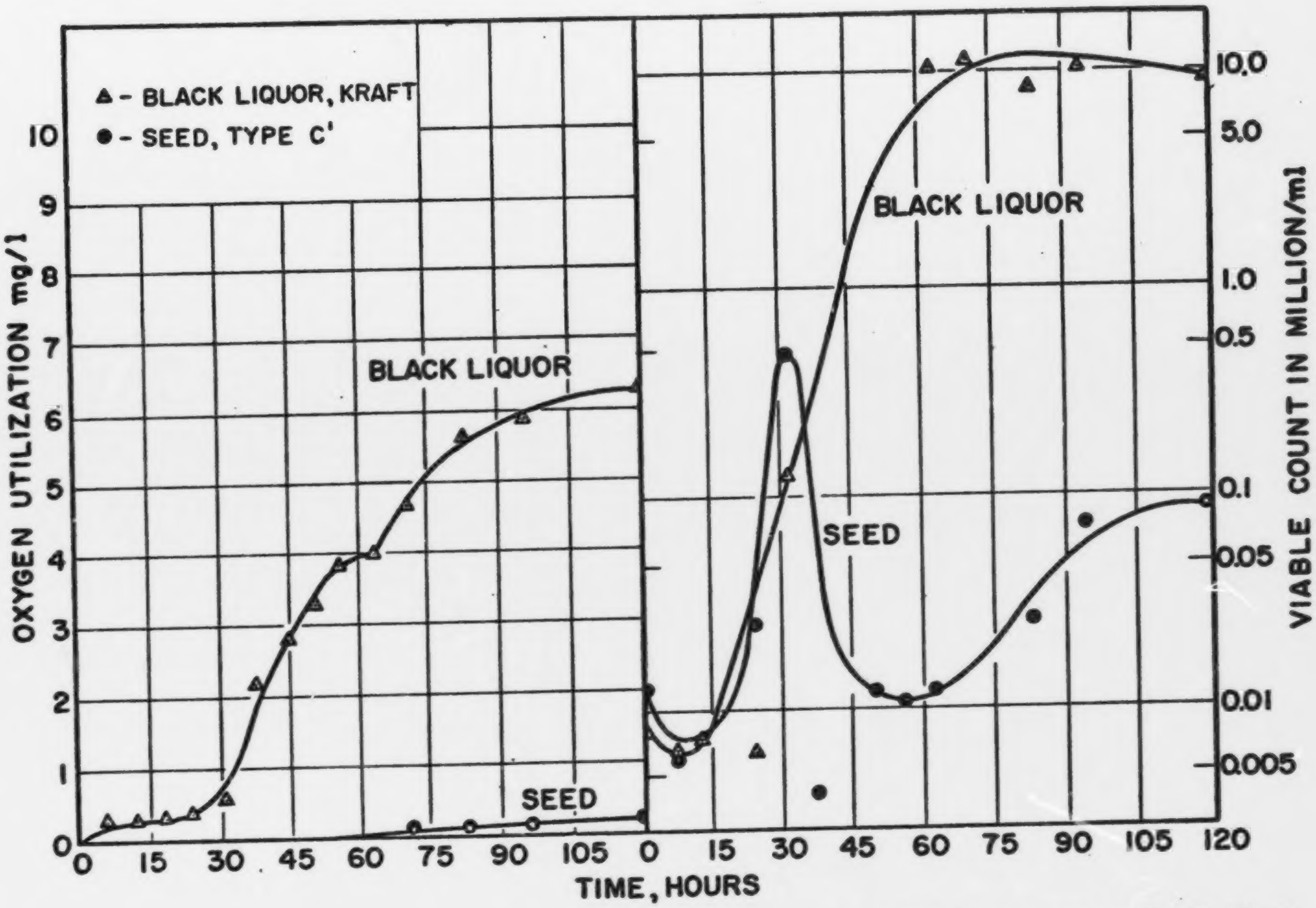

FIGURE 9. Oxygen Uptake and Viable Bacteria Count in the BOD Bottle using Kraft Black Liquor and a Washed Acclimated Seed.

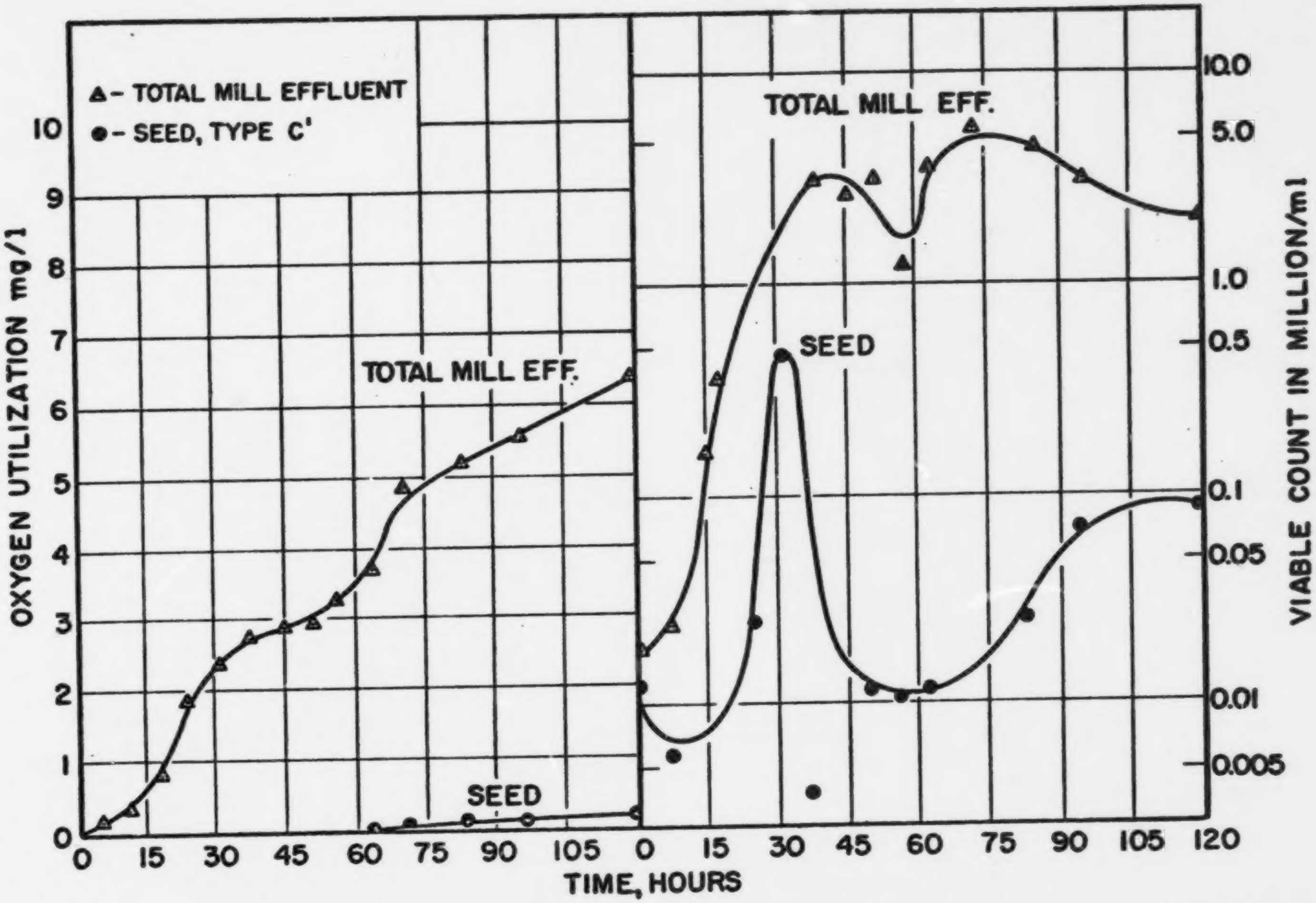

FIGURE 10. Oxygen Uptake and Viable Bacteria Count in the BOD Bottle using Kraft Mill Effluent and a Washed Acclimated Seed.

was associated with attainment of maximum population, the second stage of O_2 uptake (Figure 9) was not accompanied by a rapid dieoff in viable population. In Figure 10 there was a dieoff followed by a regrowth which corresponds to the second phase of O_2 uptake.

DISCUSSION

It was found in these studies that diphasic oxygen uptake occurred regardless of initial substrate concentration and of the type and amount of initial seed inoculum (so long as cell multiplication occurred). The results using glucose extend and substantiate the work previously reported from our laboratory. Very little work pertaining to the occurrence of the plateau has been accomplished using complex synthetic or whole wastes. The results of the complex media studies herein reported showed that either a plateau or diphasic oxygen uptake does occur for such substrates.

It is important to note that in these studies there was no alteration of the inorganic constituents included in the Standard Methods dilution water. In studies using 12 mg/l glucose it was previously stated that some doubt may exist as to a nitrogen deficiency. Rao and Busch have indicated the probability of a nitrogen deficiency at glucose concentrations above 8.06 mg/l(13). We are not in total accord with their conclusion.

Rao and Busch calculated the amount of glucose that could be optimally metabolized in the BOD bottle using standard dilution water which contains 0.445 mg/l of nitrogen. They employed the empirical formula $C_5H_7NO_2$ as the average formula weight for a biological mass and a yield factor of 41.6% conversion of substrate oxygen demand to cell material. They arrived at a value of 8.06 mg/l glucose as the upper limit for glucose concentration.

This type of estimate may be approached in other rational fashions. Assuming the formula $C_5H_7NO_2$ does reasonably well define the average composition of a bio-mass (this point itself may be subject to considerable discussion), the percentage of nitrogen in a given cell weight would be $\frac{14}{113} \times 100 = 12.4\%$. The average dry weight of a bacterial cell (normal coccus or rod) may be estimated at 2×10^{-10} mg/cell. The amount of nitrogen per cell would be: 2×10^{-10} mg/cell x 0.124 = 0.248×10^{-10} mg N/cell. The number of cells which could be produced from the 0.445 mg N/1 in the BOD bottle would be: 0.445 mg N/1 + 0.248 x 10^{-10} mg N/cell = 1.79 x 10^{7} cells/ml. It may be seen from Figure 2 that the cell count did not approach this figure. It would therefore seem reasonable to conclude that factors other than nitrogen concentration limited cell production. It is realized that the above considerations are based upon viable count, but it should also be emphasized that in the period of growth dealt with in this calculation (see Figure 2) viable count and total count usually are found to agree fairly well. There are other theoretical and experimental considerations which have a bearing upon the question of optimum nutrient concentration in the BOD bottle. However, discussion of these should be delayed until such questions as substrate partition between synthesis and respiration and the validity of using an empirical formula for bacterial cells are more clearly resolved.

The relationship between bacterial growth patterns, the plateau in oxygen uptake and the oxygen uptake phases in general found in these studies substantiated previous work accomplished in our laboratory(8)(9)(14). The duration of the plateau was again found to be correlatable with the broadness of the peak in maximum cell population. Also the behavior of the seed was found to vary; in some cases a plateau was observed in the seed systems. There appeared to be a tendency toward a more pronounced plateau at the lower substrate concentrations. The shortening of the plateau between O_2 uptake phases, and indeed its eventual disappearance as substrate concentration is increased, has been observed by McWhorter and Heukelekian in Warburg studies using much higher substrate concentrations than those employed in the BOD bottle(11). It would appear that a considerable amount of work must be done at both high and low concentrations of substrate and of cells before basic similarities between high and low energy systems can be established. For example, we cannot concur with Busch and Myrick in that "the only difference between the BOD bottle and the activated sludge process is concentration, and hence, time."(5) While it is logical to expect that the basic mechanisms of growth are the same in dilute systems as they are in more concentrated systems, the kinetic expression of these mechanisms may be of a different order as well as a different rate.

Concerning various kinetic orders and rates for exertion of BOD it is interesting to plot oxygen uptake for a typical experiment on semi-logarithmic paper. Such a plot is shown in Figure 11; the solid line represents the oxygen uptake. Phase A, according to this plot, represents that portion of the BOD curve which may be described as a first order increasing rate curve and corresponds to bacterial log growth. Following this phase the curve becomes convex upward, and if oxygen remaining is plotted (see dotted lines) the second phase (phase B) may be approximated by first order decreasing rate kinetics. This phase is followed by one in which oxygen uptake is very slight and is representative of the plateau (phase C). Following the plateau there is a phase which may be approximated as a first order decreasing rate curve (D), followed by a curve of the same order but at a much reduced velocity constant (phase E). It is seen that such a BOD curve could not be defined by one continuous mathematical expression. It has been shown by the results herein presented and by those of other researchers that phasic oxygen uptake of this type exists for BOD exertion of simple compounds and complex substrates.

For the studies using complex media, the experiments may be divided into two groups; those which exhibited a well-defined plateau, and those with only a slight plateau or simply a change in kinetics. In all of these studies it was shown that the range of maximum bacterial population corresponded either to a change in kinetics or to a plateau. The course of oxygen uptake for the nutrient broth exhibits no plateau and only a slight plateau is discernible for the neutral sulfite semi-chemical waste. Domestic sewage and the two Kraft mill wastes, however, did exhibit well-defined plateaus. Based on these studies it appears that the number of compounds in the waste has little effect on the occurrence of the plateau. Domestic sewage, which had a well-defined plateau, and the nutrient broth, which had no plateau, probably contained many more carbon sources than did the semi-chemical waste, which had only a slight plateau, or the Kraft wastes, which exhibited plateaus.

Previous work in our laboratory has lead to the formulation of four theories of causation for the plateau(9). These theories were

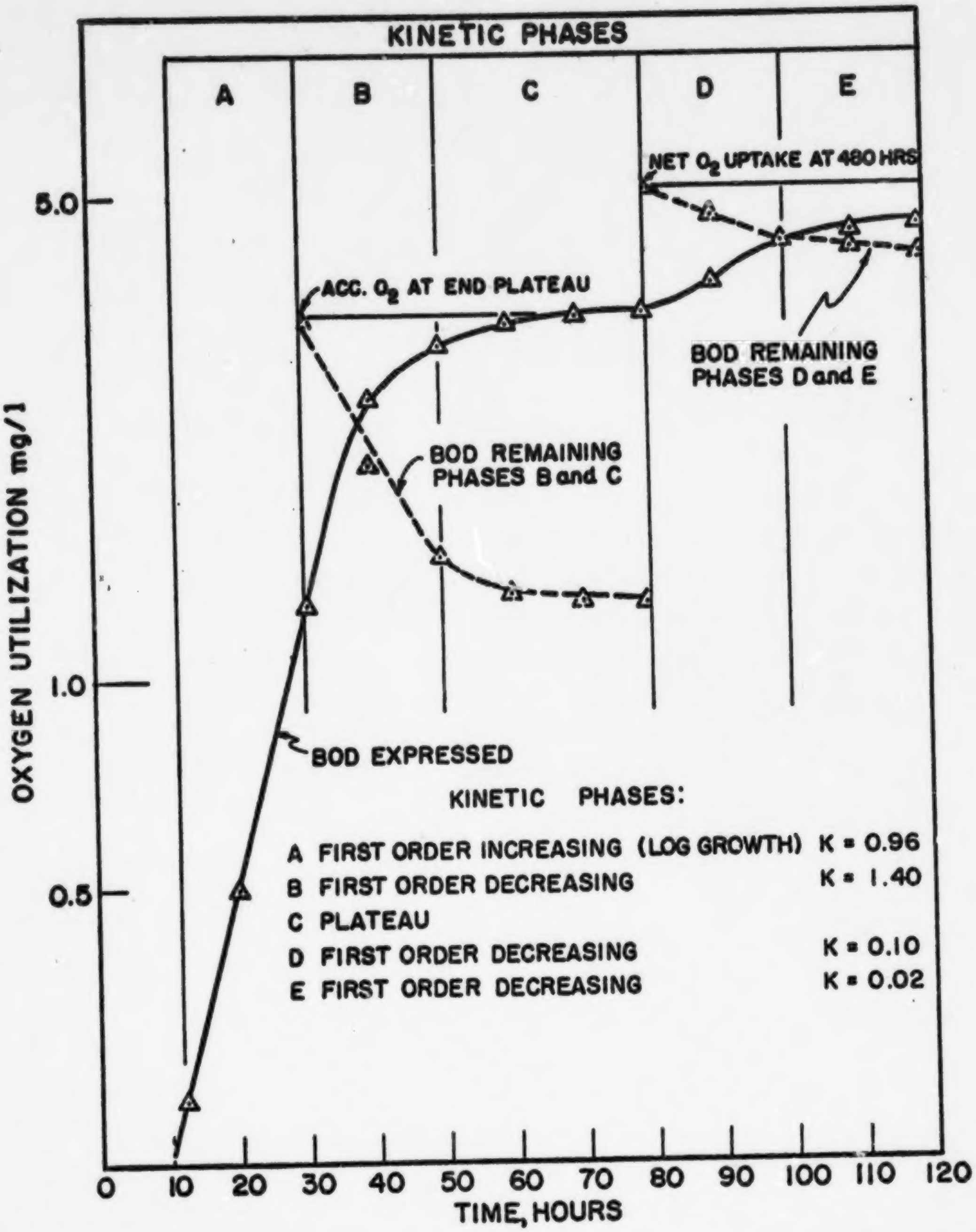

FIGURE 11. A Semilog Plot of Oxygen Uptake on 8 mg/l Glucose by an Acclimated Seed showing the Various Kinetic Phases Which may exist during BOD Exertion.

postulated based upon the relationship between bacterial growth patterns and oxygen uptake observed for many experiments. The present study was not concerned with the further delineation of generalized theories of causation for the plateau; therefore, the biochemical mechanism(s) responsible for the plateau or a change in kinetics cannot be determined beyond that which can be postulated from observed oxygen uptake and population dynamics. Since in the present studies the same oxygen-viable cell relationships were observed, the theories previously presented stand unchanged. Presently work is under way pertaining specifically to further delineation of these theories.

SUMMARY AND CONCLUSIONS

These studies verified and extended past experiments. The effect of the initial bacterial concentration and the effects of substrate concentration were studied using glucose as the sole source of carbon. The effect of complex substrates on the occurrence of the plateau was also studied for a variety of media. From these studies the following conclusions have been drawn:

1. Based upon these studies, the nitrogen concentration in the standard BOD test is not necessarily a limiting factor at a glucose concentration of 12 mg/l.

2. Phasic oxygen uptake in the BOD bottle occurred in all systems tested; however, the plateau between phases was not evidenced for nutrient broth.

3. Based upon results using the particular complex substrates employed in these studies, it would appear that the number of substrates in a medium has little or no effect upon the occurrence of a plateau.

4. In these studies the initial seed concentration had little or no effect on the occurrence of the plateau.

5. At initial seed populations which allow an increase in cell numbers, the first phase of oxygen uptake corresponds in general to a log growth phase of cell multiplication. The change in kinetics or the plateau corresponds to the attainment of maximum population density. The duration of the plateau depends upon the length of time that the cell numbers remain near the maximum population. The second phase of oxygen uptake corresponds to a dieoff of the cells.

ACKNOWLEDGEMENT

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PAPER 8

FACTORS AFFECTING THE EXISTENCE OF THE PLATEAU DURING THE EXERTION OF BOD

By

A. F. Gaudy, Jr., M. N. Bhatla, R. H. Follett, and
F. Abu-Niaaj

This paper summarizes results presented in Papers 4, 5 and 7 and was published in the Journal Water Pollution Control Federation, 37, 444 (1965). (See note, Paper 4.)

FACTORS AFFECTING THE EXISTENCE OF THE PLATEAU DURING THE EXERTION OF BOD

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Since its inception during the latter part of the nineteenth century, the BOD test has been the subject of much controversy and continuing research. It is used as a parameter in research, and it is also the subject of research. It is used as a "control" parameter for treatment plants; it is also a major basis for regulatory action. It is an integral part of dissolved oxygen sag curve analysis and it forms a major basis for design of treatment units; yet, as stated by Hoover, Jasewicz and Porges, "no one appears to consider it adequately understood or well adapted to his work" (1). The BOD test is indeed the paradox these authors and many others have considered it to be. Perhaps the fact that it has been called upon to serve such universal duty helps explain in some measure why it cannot serve adequately all the purposes to which it has been put.

It would appear that until fairly recent times the major research effort involved proving that the kinetics of carbonaceous BOD expression were first order decreasing rate, while during the past 25 or 30 years research efforts have been centered around showing that the first order decreasing rate concept does not suffice to depict BOD kinetics. An examination of the literature shows the major kinetic concepts which have been applied to BOD exertion. The concept first held and most prevalently used is the first order, continually decreasing rate type of kinetics which prevailed in the studies made by Theriault (2). It has been stated many times that there is no fundamental reason to expect such kinetics. This is, however, a convenient way to express the course of BOD exertion, since only two constants, L and k, adequately describe the curve. Thus, in an engineering sense, it is highly desirable. From a theoretical standpoint, this type of kinetic curve might obtain in a system in which a limiting metabolite controlled the course of reaction. For example, biological systems devoid of an extracellular source of nitrogen (nonproliferating cell suspensions) have been observed to yield this type of oxygen uptake curve.

Oxygen uptake curves best described as "autocatalytic" have also been observed in the BOD bottle (3). The term "lag" has been applied to describe the early portion in which the curve is concave upward. It is recognized that this is not necessarily a lag in bacterial activity; however, in order to use the first order decreasing rate expression, it must be treated as one. When the increasing rate portion is of short duration, the lag equation of Thomas would appear to provide a satisfactory treatment of the kinetics (4). It is emphasized, however, that this increasing portion of the curve may often be shown to be first order increasing rate. With an acclimated seed, this type of kinetics would be expected if the system were started using a low initial inoculum. The upsweeping portion would then correspond to a log growth phase and not a lag phase.

A third type of oxygen uptake curve for BOD exertion has been observed which can best be described as two-phase, first order decreasing rate, wherein a rapid phase is followed by a slower one (1)(5). In addition, there may be more than two first order portions. This type of kinetics might be expected with somewhat high initial seed inocula in which the carbon source is not sufficient to support much new growth (as viable population increase), thereby preventing an autocatalytic curve. The separation of oxygen uptake into two phases has been explained as "rapid assimilation of substrates into carbohydrate storage products followed by a slow endogenous utilization of these intracellular products" (1). As early as 1926 Greenfelder and Elder noted a second stage, and suggested that it could be due to the death of organisms which were living at the time of sampling, and which, after dying, would become part of the carbonaceous oxygen demand (6).

Recently Busch has observed another type of carbonaceous curve in which the rapid phase is followed by a second stage, but the two stages of oxygen uptake are separated by a plateau (7). After the plateau, oxygen utilization in excess of endogenous respiration is attributed to the activity of the protozoa and other predators which feed on the bacteria. Except for the involvement of predators, the theory as stated by Busch is similar to that of Hoover, et al. (1). The difference lies in the plateau or pause in oxygen uptake before initiation of the second stage. Busch has suggested that "as the ratio of predators to substrate-consuming population increases, the separation of the two stages begins to blur" (7). He observed a reduced oxygen uptake after treatment of the seed to reduce the protozoan population. Wilson and Harrison have objected to Busch's conclusion that the rise in the second stage represented the joint effects of endogenous respiration and predator activity (8). To test Busch's hypothesis they performed an experiment using a pure bacterial culture and phthalic acid as substrate. In their experiments oxygen uptake was measured on two systems containing different amounts of cell inoculum. They felt from their results that it was "apparent that the magnitude of the second stage of oxygen uptake depends solely upon the number of cells present" (8).

McWhorter and Heukelekian have also investigated the occurrence of the plateau in oxygen uptake measured in Warburg respirometers (9). They varied substrate concentration (100, 500, 1000, 2000 mg/l glucose), and used 0.5 ml settled sewage as seed. Potassium nitrate was used as a nitrogen source. They found that the lag between phases (or the plateau) was shorter for 500 mg/l glucose than for the 100 mg/l concentration. The lag did not appear in the 1000 and 2000 mg/l systems. For the two systems exhibiting a plateau, they observed that while the increase in substrate concentration increased oxygen uptake, the accumulated uptake at the plateau expressed on a relative basis (per percent theoretical) was the same for both systems. They also investigated the effect of predators and concluded that the disappearance of the plateau with increasing substrate concentration could not be attributed solely to changes in the predator ratio, since O_2 uptake curves for sonicated and normal seeds did not diverge until 45 per cent of the theoretical demand had been expressed. This was above the plateau value which both they and Busch had obtained.

From the above review it can be seen that there is considerable interest in this newest type of kinetic expression for carbonaceous BOD, i.e., the diphasic curve with the two phases separated by a discernible pause or plateau. It can also be seen that there is some controversy concerning the generality of its occurrence, and that there are no generally satisfactory explanations for its occurrence. In reporting on their pure culture studies, Wilson and Harrison stated that the plateau might be caused by the delayed onset of endogenous respiration, but offered no explanation as to why endogenous respiration should be so delayed. Since the plateau occurs in both heterogeneous and pure culture populations, its presence cannot be solely attributed to changes in predominance (bacterial predominance or a change from bacteria to predators).

In considering the five-day BOD test, the occurrence of the plateau is extremely important since it usually occurs within 48 hours of incubation. Depending upon the length or severity of the plateau, it could seriously affect the 5-day BOD value. Its existence also precludes the employment of any simple kinetic expression for predicting the course of carbonaceous BOD exertion. It appeared to the authors that there were two major problems or questions concerning the plateau which warranted investigation:

1) Is the plateau of general occurrence, or does it appear only under certain unique experimental conditions? For example, would it depend on type of seed, or pre-treatment of seed, type and/or concentration of substrate, complexity of the waste, etc.? These are important aspects since if, for example, the plateau occurred only for specific pure compounds or for a limited mixture of compounds, its significance in water pollution control would be slight and the expenditure of experimental effort in this area would be of limited value.

2) Why does the plateau occur? Are there sound, theoretical

explanations for its occurrence? Is there any one general reason, or are there many? If there is more than one basic explanation, which one is the most prevalent cause in water pollution control work? These aspects also had important ramifications for work currently under way in the authors' laboratory concerning the kinetics and mechanism of substrate removal. The studies herein presented were undertaken primarily to determine the generality or constancy of the occurrence of the plateau under various conditions of seeding, using a wide array of seeding materials and substrates. In accomplishing these experiments, over 50 long term BOD studies were made in which both oxygen uptake and numbers of viable bacteria were determined. The cell count was followed in an effort to gain an insight into causation for the plateau. The purpose of the present report is to show representative results which illustrate conditions under which the plateau occurs, and which allowed formulation of theories of causation for the occurrence of the plateau. Portions of this work have been presented at two research conferences (10)(11).

MATERIALS AND METHODS

Throughout the investigations BOD and dissolved oxygen reagents were those prescribed in Standard Methods for the Examination of Water and Wastewater (12). Dissolved oxygen was measured using the Alsterberg modification of the Winkler method. BOD's (seed blank and samples) were run using various substrates, types of seed, and seed concentrations. At each sampling time, before adding Winkler reagents 1 ml was withdrawn from each system, appropriately diluted, and used to determine the viable bacteria count. Preliminary experiments had shown that dissolved oxygen values were unaffected by the extra manipulation required in withdrawing the sample prior to determining the dissolved oxygen. Changes in population were measured for the seed blanks as well as for the sample bottles. This was done in order to gain comparable information on both systems. In addition, this procedure provided for a partial check on the carryover of substrate with the seed. The spot plate counting technique was used to estimate the viable bacteria count (13)(14)(15).

Four types of seeding material were used. Seed type A consisted of effluent from the primary clarifier of the municipal sewage treatment plant of Stillwater, Oklahoma.

Seed type B consisted of effluent from a batch-operated laboratory activated sludge developed from an initial seed of type A and grown on the particular carbon source or waste under investigation.

Young cells (seed type C) were developed from an initial inoculum of seed type B. They were grown on the particular carbon source under study. The cells were harvested by centrifugation near the end of the log growth phase and washed three times with 0.05 M phosphate buffer.

Seed type D consisted of a pure culture, prototrophic strain of Escherichia coli, grown on a shaker apparatus, harvested near the end of the log growth phase and washed as in seed type C. All experiments using seed type D were conducted under aseptic conditions.

Seed type B was grown on a synthetic waste composed of (concentrations in mg/l): carbon source as COD, 1000; $(NH_4)_2SO_4$, 500;

$MgSO_4.7H_2O$, 100; $FeCl_3.6H_2O$, 0.5; $MnSO_4.H_2O$, 10; $CaCl_2$, 7.5. These components were dissolved in 0.01 M phosphate buffer, pH 7.0, containing 100 ml of tap water per liter.

Seed types C and D were grown in a medium consisting of the same constituents as those listed above, except that the carbon source was increased 5-fold, salts 2-fold, and buffer 3-fold. This was done in order to insure a large harvest of cells and to prolong the log growth phase. Previous experience had indicated that the limiting metabolite for such a synthetic waste was the carbon source.

Among the pure compounds used as substrates in these studies were glucose, glycerol, glycogen, lactose, ribose, sodium glutamate, sorbitol, melibiose, and galactose. Two-component systems consisting of glucose + ribose and glucose + sorbitol were also investigated. Among the complex media used in these studies were nutrient broth, sterile sewage, neutral sulfite semichemical pulp liquor, Kraft mill digester liquor, and Kraft mill effluent.

RESULTS

Since the plateau was evidenced for most of the substrates and seeding conditions examined, graphical presentation of all the results does not seem necessary and is therefore limited to presentation of figures which demonstrate the occurrence of the plateau and bacterial growth patterns for a sufficient number of seeding conditions and substrates to show the basis for the theories of causation postulated in the discussion. Pertinent information for all 51 experiments is given in Table I. In 8 of 51 experiments no plateau was observed; however, there did not appear to be any one critical variable which precluded the occurrence of the plateau.

TABLE I

SUMMARY OF LONG-TERM BOD STUDIES OF FACTORS AFFECTING BOD KINETICS

Exp.No.	Substrate	Concentration mg/1	Seed Type	Initial Concentration Cells/mlx10-6	Type Kinetics[1]	% Theoretical[2]
1	Glucose	8	A	0.02	Plateau	31
2	Glucose	5	A	0.5	Plateau	38
3	Glucose	9	B	0.07	Plateau	33
4	Glucose	4	C	2	Plateau	35
5	Glucose	6	C	6	Plateau	31
6	Glucose	10	C	4	Plateau	37
7	Glucose	4	C	4	Plateau	30
8	Glucose	4	D	12	Plateau	35
9	Glucose	8	D	1	Plateau	34
10	Glucose	4	D	1	Plateau	37
11	Glucose	8	D	20	Plateau	32
12	Glucose	8	D	20	Plateau	33
13	Glucose	8	D	0.01	Plateau	35
14	Glucose	8	D	12	Plateau	35
15	Glucose	4	C	3	Plateau	33
16	Glucose	4	C	2	Plateau	34
17	Glucose	4	C	2	Plateau	43
18	Glucose	4	D	20	Diphasic	26
19	Glucose	4	D	12	Plateau	37
20	Glucose	8	B	0.01	Plateau	39
21	Glucose	5	B	0.01	Diphasic	36
22	Glucose	2	B	0.01	Plateau	51
23	Glucose	12	B	0.02	Plateau	34
24	Glucose	12	B	0.0005	Plateau	32
25	Glucose	8	C	0.002	Plateau	41
26	Glucose	8	C	0.1	Plateau	40
27	Glucose	8	C	5	Plateau	32
28	Glucose	8	C	10	Single Phase	-
29	Glucose + Ribose	4+4	C	2	Plateau	30
30	Glucose + Sorbitol	4+4	C	2	Plateau	36
31	Glucose + Sorbitol	4+4	C	0.2	Plateau	35

Exp.No.	Substrate	Concentration mg/1	Seed Type	Initial Concentration Cells/mlx10-6	Type Kinetics[1]	% Theoretical[2]
32	Glucose + Sorbitol	4+4	D	20	Plateau	37
33	Glucose + Sorbitol	4+4	D	12	Multiphasic	41
34	Sorbitol	10	C	3	Plateau	34
35	Sorbitol	4	C	2	Plateau	35
36	Sorbitol	4	C	0.2	Plateau	36
37	Sorbitol	4	D	20	Diphasic	35
38	Sorbitol	4	D	12	Plateau	40
39	Ribose	8	C	3	Plateau	32
40	Ribose	4	C	2	Plateau	30
41	Glycerol	8.5	C	-	Plateau	43
42	Glycogen	8	C	2	Diphasic	38
43	Lactose	8	C	3	Plateau	38
44	Glutamate	10	C	9	Plateau	40
45	Melibiose	10	C	5	Plateau	25
46	Galactose	8	C	10	Diphasic	27
47	Nutrient Broth	1.9ml/1	A	0.004	Diphasic	-
48	Sewage	40 ml/1	A	0.002	Plateau	-
49	Neutral Sulfite Liquor	0.2ml/1	C	0.02	Plateau	-
50	Kraft Black Liquor	-	C	0.01	Plateau	-
51	Kraft Mill Effluent	-	C	0.02	Plateau	-

[1] Plateau - two phases separated by a distinct pause of variable length; diphasic - two phases not separated by a pause; single phase - "normal" BOD curve; multiphasic - more than two distinct rates, not separated by pause.

[2] % Theoretical: oxygen used in the first phase of uptake calculated as a per cent of the theoretical total oxygen demand of the substrate.

Figure 1 shows the course of BOD exertion and changes in the viable population for systems containing 8 and 5 mg/l of glucose using municipal sewage seed (type A). Multiphase kinetics were exhibited in both experiments regardless of the initial concentration of viable cells or substrate. In both experiments there is a definite evidence for the plateau in BOD exertion. The first, or rapid, phase of oxygen uptake corresponds to the log phase of growth. The plateau occurs in the region of maximum viable count, and the last stage of oxygen uptake corresponds to a sharp decrease in viable population. The behavior of the seed system is of particular interest. With a low initial inoculum (2×10^4) the viable count in the seed experienced a considerable log phase (approximately six doublings of the population), whereas in the other experiment there was an initial decrease in viable cells followed by a slight increase which in approximately 70 hours yielded a population density equal to that of the initial inoculum. The seed behavior may be attributed to the difference in viable population in the sewage seed, and to the variable strength of organic matter in the sewage seed from which the seed was obtained.

Results for an experiment using seed obtained from the effluent of a laboratory activated sludge plant operating on glucose with sludge developed from an initial inoculum of municipal sewage (type B) is shown in Figure 2. In this case there was very little oxygen used in the seed system. The population remained static during the first 120 hours, then gradually fell off. The system containing substrate showed the full gamut of BOD kinetics; there was a slight lag followed by an autocatalytic portion. The plateau was again in evidence, and corresponded to the range of maximum population density.

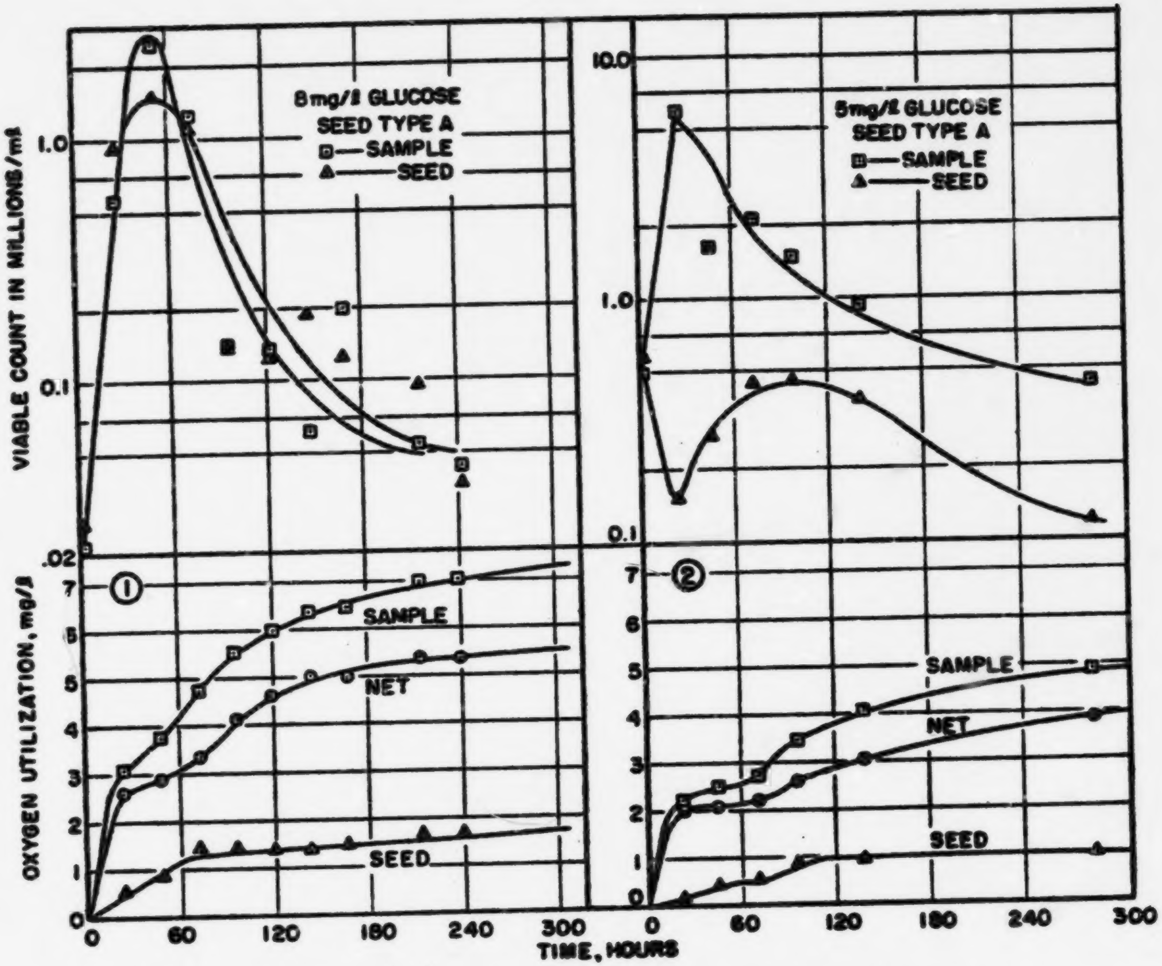

FIG. 1 - BOD AND VIABLE BACTERIA COUNT, SEED TYPE A

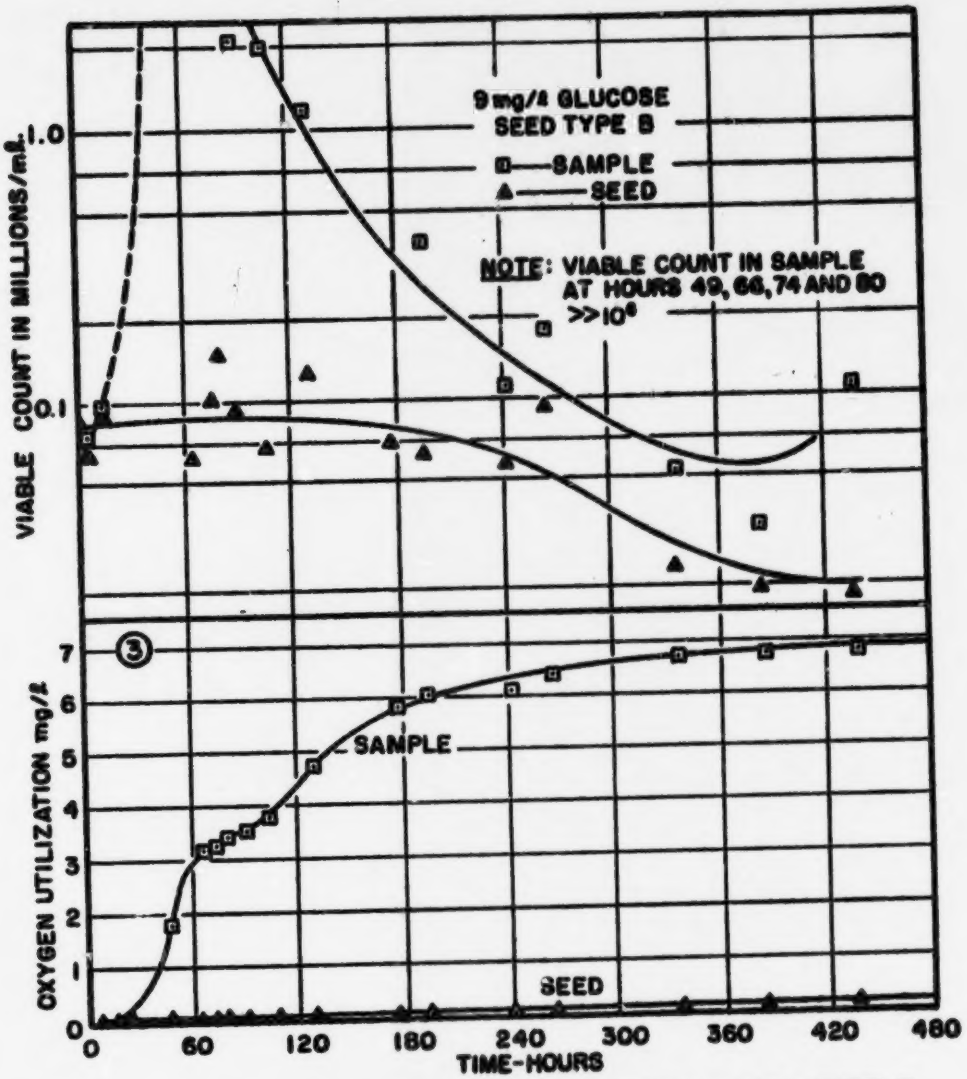

FIG. 2 - BOD AND VIABLE BACTERIA COUNT, SEED TYPE B.

Figure 3 shows results using washed young heterogeneous seed developed from effluent of the laboratory activated sludge unit. Experiments are shown for glucose concentrations of 4, 6, and 10 mg/l. The plateau was evidenced in all experiments. It was so pronounced in the experiment shown at the left of the figure that subtraction of the seed yielded an apparent decrease in accumulated oxygen uptake in the sample bottle. In all cases the plateau occurred in the region of maximum population, and the last stage of BOD exertion corresponded to a dieoff in the viable population.

For the experiment shown in Figure 4, Escherichia coli was used as seeding material (type D). The figure shows results for high and low initial seed populations. In both cases a plateau was observed. It is seen that at the low seed concentration, even though the seed was washed three times, enough substrate was carried over to provide for approximately six doublings of the population. During this time there was no discernible oxygen uptake in the seed system, as measured by the Winkler method. Oxygen uptake in the seed did not occur until the dieoff of bacterial population was under way. It can also be seen that the plateau in the BOD exertion curve again occurred in the range of maximum viable population.

In further efforts to determine the patterns and generality of the occurrence of the plateau, BOD experiments were undertaken using seed grown and harvested in the same manner as seed type C, except that the cells were grown not on glucose, but on the particular carbon source studied. Results of eight such experiments are shown in Figure 5. In order to present all eight experiments in one figure, thus facilitating comparison of the systems, it was

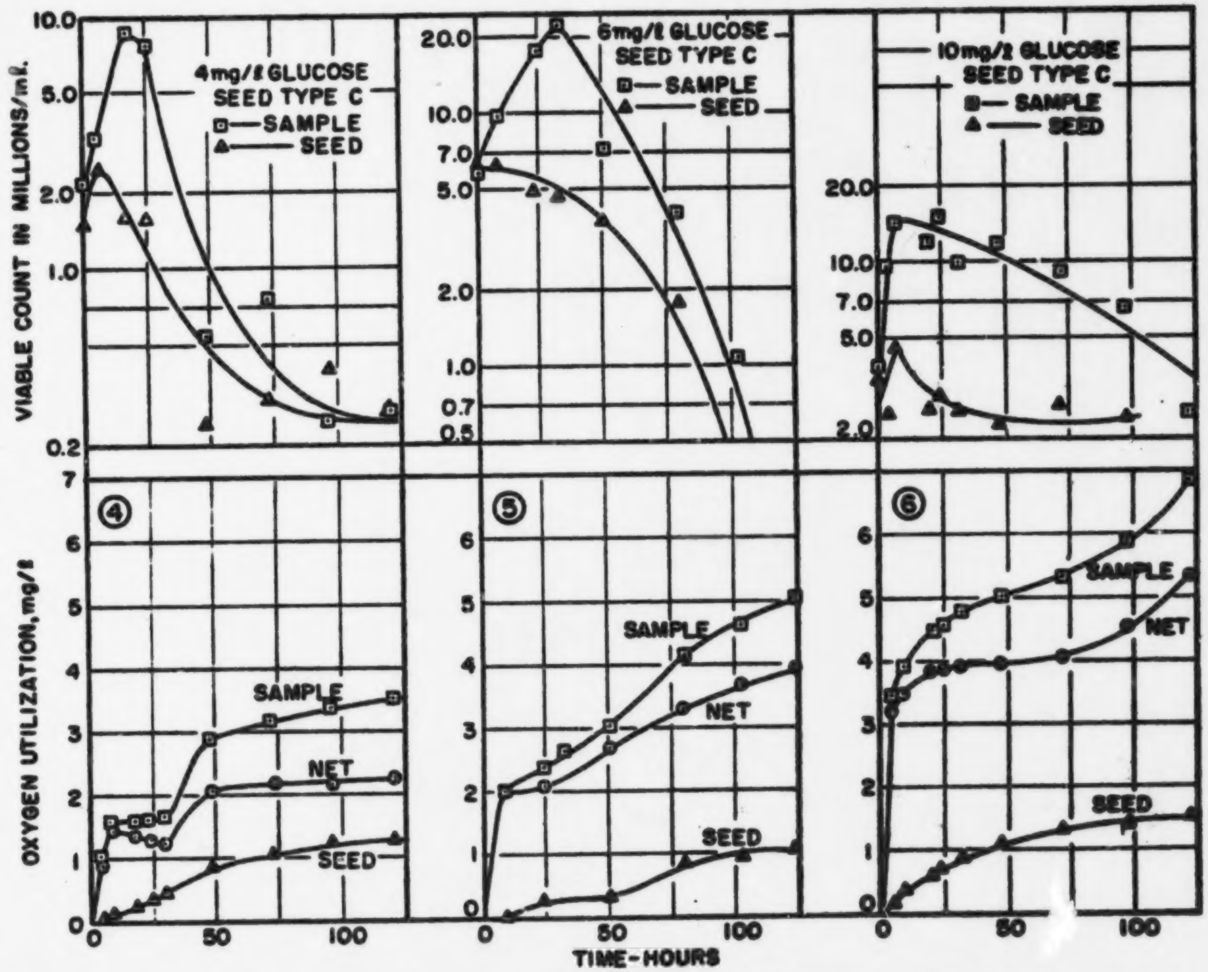

FIG. 3 - BOD AND VIABLE BACTERIA COUNT, SEED TYPE C.

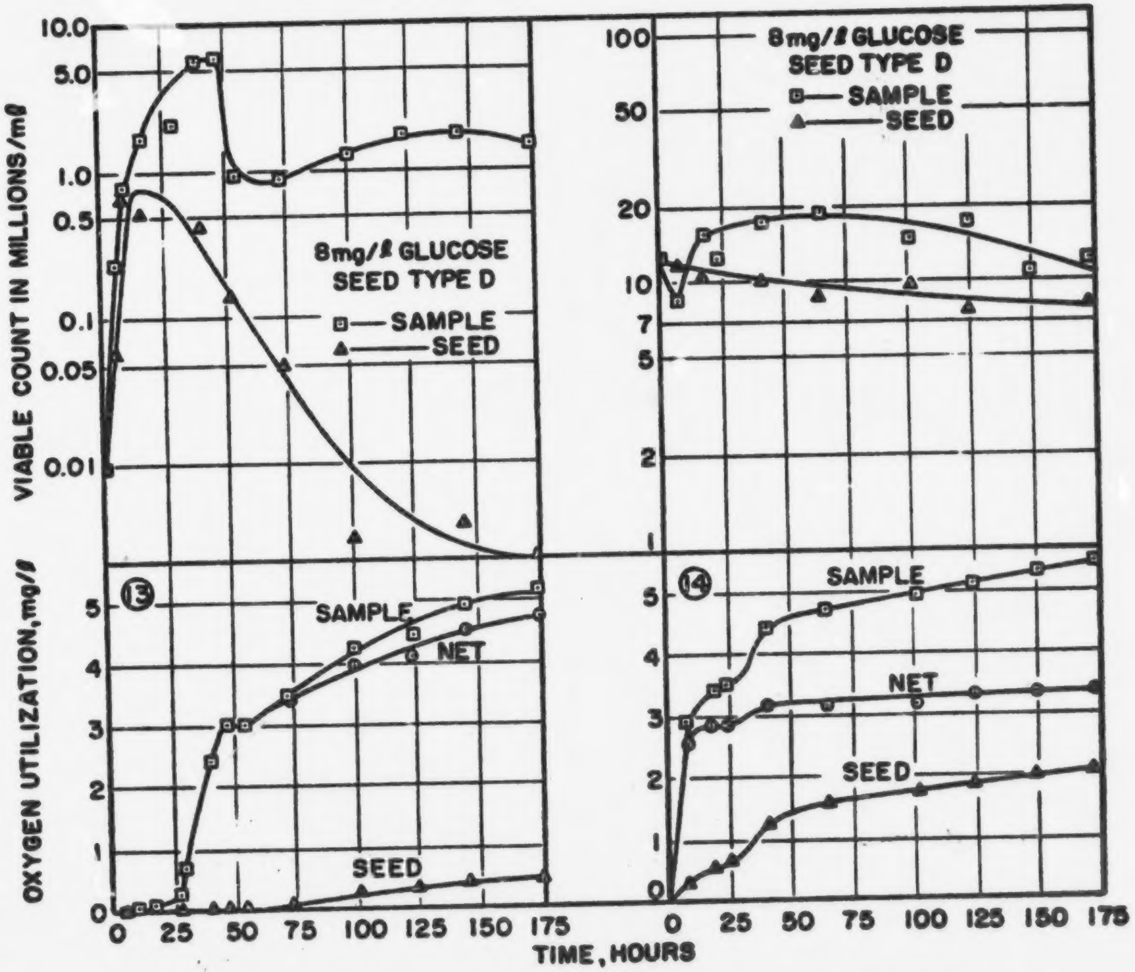

FIG. 4 - BOD AND VIABLE BACTERIA COUNT, SEED TYPE D.

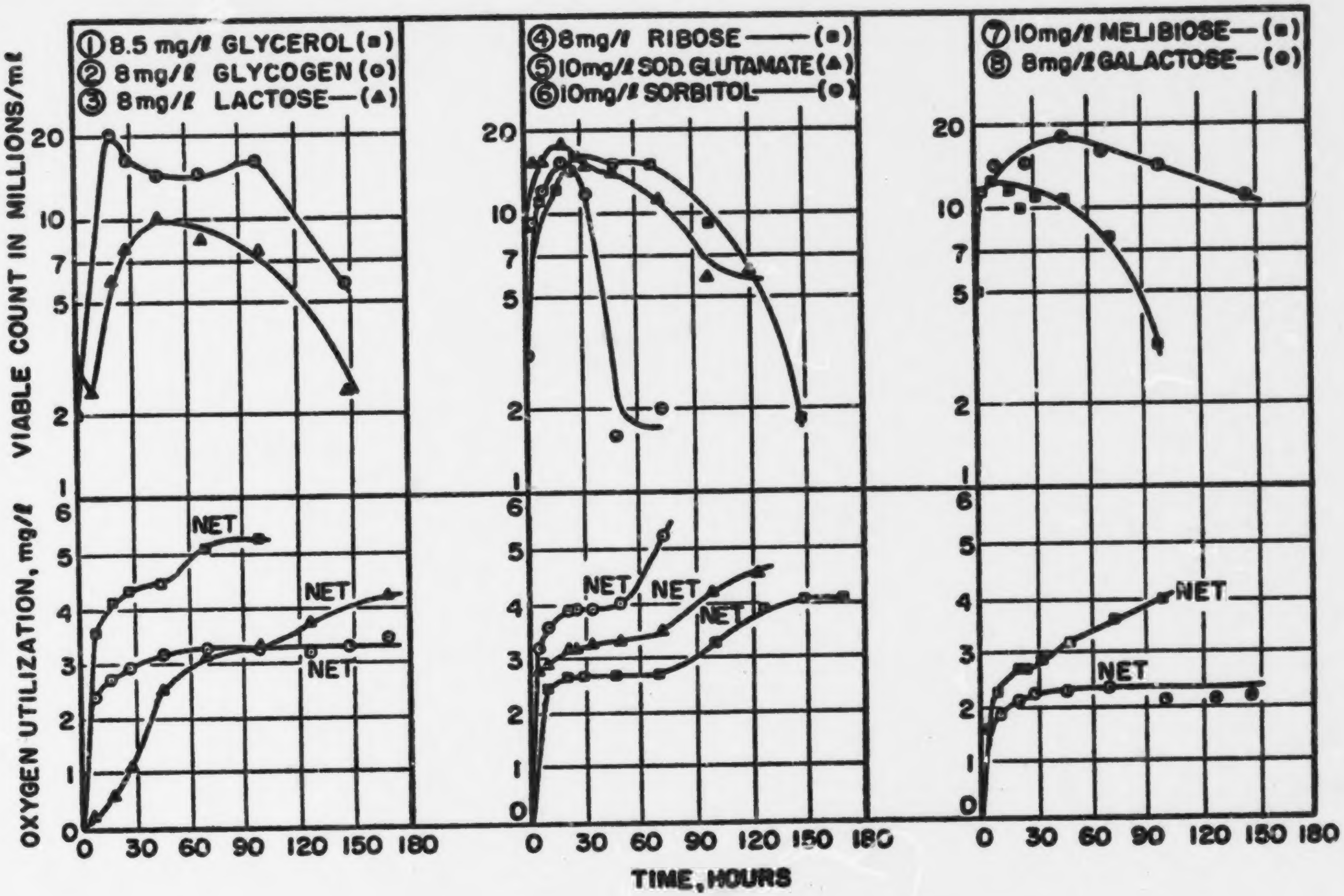

FIG. 5 - BOD AND VIABLE BACTERIA COUNT, SEED TYPE C.

impossible to show oxygen uptake and population dynamics for the seed; therefore only net oxygen uptake and the viable count in the sample bottles are shown. Viable count for the glycerol system (experiment 1, Figure 5) was not obtained. It is seen that there was definite evidence of a plateau in net oxygen uptake for all systems except glycogen and galactose (experiments 2 and 8); however, the total oxygen uptake curve for these experiments (not plotted) showed a slight plateau which corresponded to one in the seed. Subtraction of the oxygen uptake of the seed yielded a net curve with no plateau in the glycogen system, whereas for galactose the second stage uptake in the seed was more abrupt, and upon subtraction yielded a net curve which shows a negative oxygen utilization (see last three points, experiment 8). Oxygen uptake of the seed for lactose, ribose, glutamate, sorbitol, and melibiose was also diphasic, but did not mask the plateau in net oxygen uptake.

The total oxygen uptake for glycerol gave only a slight indication of a plateau, but the seed uptake broke so sharply that it accentuated the plateau in net oxygen utilization. In all experiments shown in Figure 5 in which a plateau was observed it is seen that, in general, the plateau region corresponded to the region of maximum population density. The longer the pause in viable count before the dieoff, the longer was the plateau.

Figure 6 shows the course of BOD exertion and viable count for a synthetic waste consisting of 4 mg/l glucose + 4 mg/l ribose. Control systems for glucose and ribose are also shown. The seed was grown on glucose and harvested according to the procedure described for seed type C. All systems, including the seed, exhib-

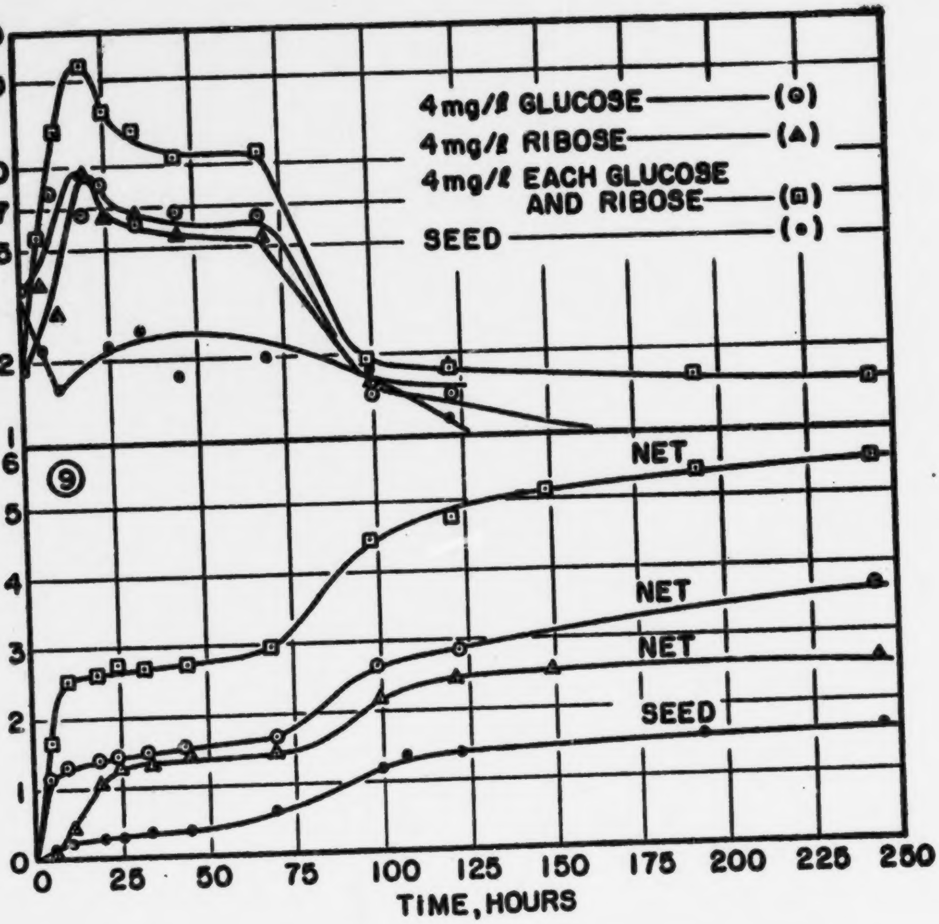

FIG. 6 - BOD AND VIABLE BACTERIA COUNT, SEED TYPE C
ACCLIMATED TO GLUCOSE

ited phasic oxygen uptake. Acclimation to ribose took place in less than six hours, as seen from the ribose control system, which also shows a plateau at approximately 25 hours. The glucose control and the combined system also exhibited a plateau which occurred at approximately 10 hours. Again, the same relationship between oxygen uptake and viable population was observed.

Sewage obtained from the primary settling tank effluent of the Stillwater municipal treatment plant was employed as substrate at a dilution of 40 ml/l of the dilution water for the experimental results shown in Figure 7. The portion employed as substrate was sterilized before use, while an unsterilized portion (0.25 ml/l dilution water) of the same sewage sample was used as seeding material. These results demonstrate the full gamut of BOD kinetics typified by two phases separated by a plateau. As for experiments using defined substrates, the plateau corresponds to the range of maximum population density, and the second stage is associated with the decrease in viable population.

DISCUSSION

Out of a total of 51 experiments undertaken in this phase of the investigation, the plateau was evidenced in 43. It was observed to occur over a fairly wide range of simple and complex substrates and with various concentrations and types of seeding materials (from the most heterogeneous which might be expected, i.e., sewage seed, to a pure culture seed of Escherichia coli). It was found that the plateau generally occurs within the first two days of incubation, and more often within the first day. Usually 30 to 40 per cent of the theoretical oxygen demand of the

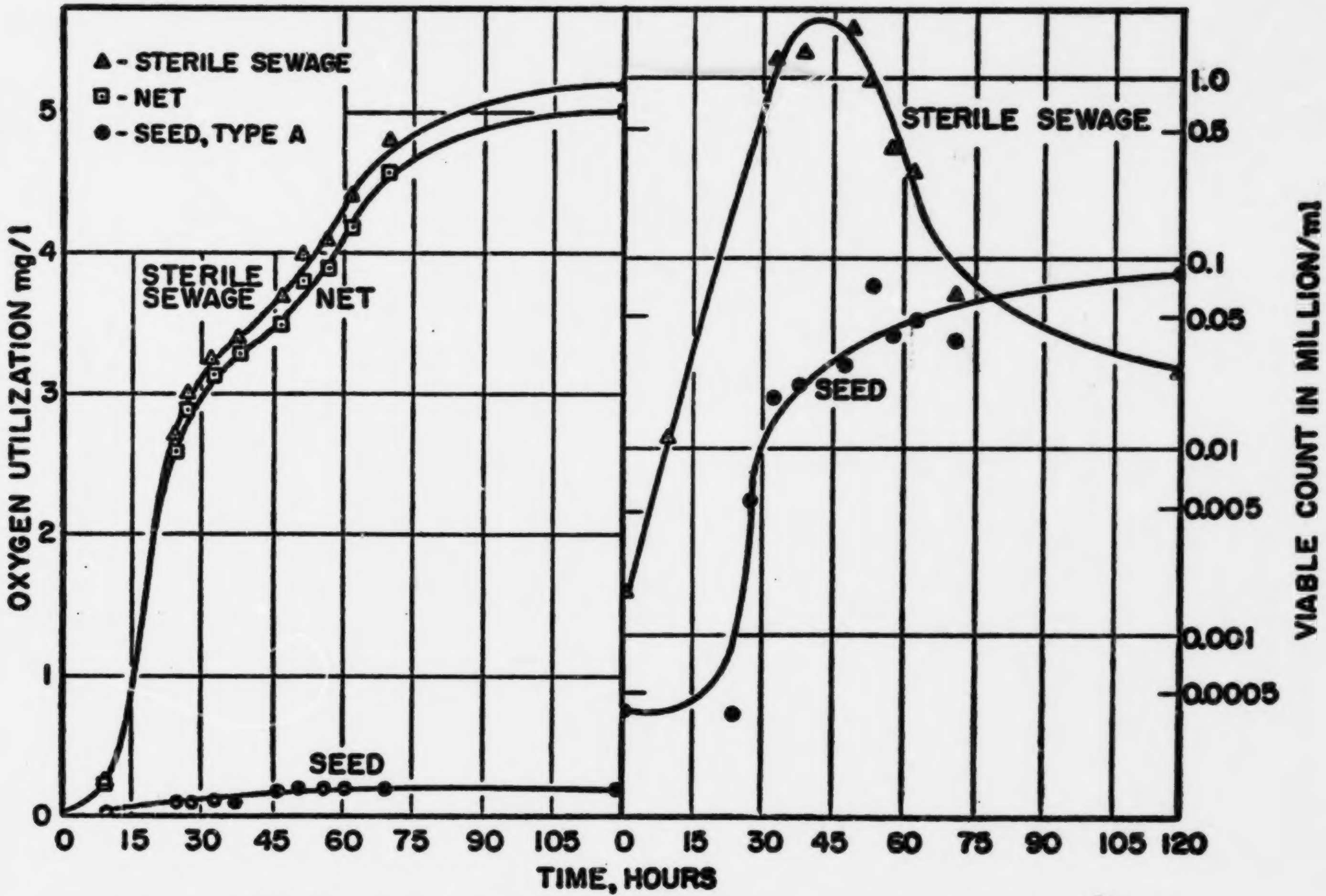

FIG. 7 - OXYGEN UPTAKE AND VIABLE BACTERIA COUNT IN THE BOD BOTTLE USING STERILE SEWAGE AND A SEWAGE SEED

substrate (for cases where such a calculation is possible) had been exerted at the time of occurrence of the plateau, but some values below and above this range were observed. For some experiments the length of the plateau was very short, and in a practical sense might not seriously affect prediction of the kinetic course of BOD exertion. In other cases, however, the plateau was very extended and would thus seriously affect a prediction of the course of carbonaceous BOD expression, and would also affect the 5-day BOD value as well. It should also be noted that the plateau was observed in many seed systems. From these findings it can be concluded that the plateau is a fairly common occurrence, but the geometry of BOD curves which exhibit plateaus cannot yet be defined with enough precision to warrant the use of the plateau for predicting the course of BOD removal. It would appear that the ultimate utility of the plateau value will depend not only upon adequate formulation of predictable geometry of the BOD curve, but also on the development and experimental proof of theories explaining the mechanism of causation for the occurrence of the plateau.

There is a need for general agreement in the field concerning the use of the BOD test. It was stated at the outset that the BOD test has been called upon to serve many functions and, indeed, this has led to many interpretations of BOD data. More often than not it is used as a test for removal of substrate (or organic matter). If this were its only use and if the exogenous substrate were removed by the time of the occurrence of the plateau, a short term test using the plateau value could be devised. However, this is not the originally intended use of BOD data, since it is intended as a measure of the oxygen required to stabilize organic matter and some of the oxygen is used after the original substrate is removed. Also, if BOD data are to be used to help predict occurrences in the receiving stream, the kinetic course of the entire carbonaceous BOD curve must be adequately formulated. It can be seen that in light of the plateau and the kinetic discontinuity it causes, the BOD test cannot serve both broad purposes.

In addition to investigating the scope and conditions for occurrence of the plateau, the present studies were designed to gain some insight into causation for its existence, and for this reason bacterial count curves were obtained in each experiment. It was found in these studies that the following general correlation existed between oxygen uptake and viable count:

1) The rapid rise in oxygen uptake during the first stage corresponded to rapid bacterial multiplication.

2) The plateau in oxygen uptake corresponded to the peak range of population density, and the duration or severity of the plateau was related to the period of time in which the population remained at or near the maximum density.

3) The second stage of oxygen uptake corresponded to a decreasing portion of the bacterial count curve.

One of the prevalent theories of waste purification states that rapid oxygen uptake represents incorporation of all substrate into storage products and/or new cells, and the last phase represents endogenous respiration of stored products. If this theory holds in dilute suspensions, the dieoff of the bacterial population observed in the present experiments could be interpreted as dieoff only in the sense that the cells are no longer capable of replication, but can still respire. In systems where protozoa are known to exist, a bacterial dieoff accompanied by a second stage of oxygen uptake can be partially attributed to ingestion of bacteria as food for protozoa. In the work herein presented the plateau was not as pronounced for pure culture systems as it was for most of the heterogeneous systems. However, based upon work using this particular pure culture, it is obvious that the second stage of oxygen uptake can be due to reasons other than the presence of protozoa. In addition, the magnitude of oxygen uptake in the second phase does not, from the present studies, appear to "obviously depend upon the number of cells present," as suggested by Wilson and Harrison (8). Indeed, comparison of the two experiments shown in Figure 4 indicates that the magnitude of the second stage was greater in the system containing fewer viable bacteria. The fact that the plateau occurs in both pure culture and heterogeneous systems indicates either that there may be more than one valid cause for the plateau, or that there is a basic biochemical reason for its existence which is independent of the species exerting oxygen uptake.

Other investigators have studied changes in bacterial population and oxygen uptake during the utilization of organic carbon sources in dilute systems. Butterfield studied oxygen uptake and viable count using B. aerogenes and a small sewage coccus (16). For these particular pure cultures there was no appreciable dieoff of cells in the 160-hour experiment; also Butterfield's data showed no evidence of a plateau. The shape of the oxygen uptake curve was the same as that of the viable count curve.

Lea and Nichols have also followed the course of carbonaceous BOD exertion and viable count (17). Daily sampling over a 5-day period showed that the bacterial count curve had essentially the same shape as the BOD curve. The viable count increased steadily at a decreasing rate, reaching the maximum population at the close of the experiment, five days.

In both of the works cited above there was no evidence of a plateau in BOD exertion. More recently Lee and Oswald have traced the course of BOD exertion and changes in viable population (18). Their data showed a rapid rise in bacterial count, with the maximum population being reached in one to 1½ days. The oxygen uptake rate was also determined for 2 and 6-hour intervals. These data showed an extremely rapid uptake rate followed by a sharp decrease. The maximum rate occurred during the first day; that is, slightly before the peak in viable count. No plateau was indicated in their accumulated uptake curve (corrected for a lag period). However, the rate curve shown in one of the two experiments presented would seem to indicate a rapid rise followed by a plateau and a second stage of oxygen uptake. The rapid phase of oxygen uptake was attributed to cell growth; however, they considered it probable that although cell division took place, the cells remained in clumps or flocculent masses, and therefore the recorded count would be less than the actual count. A rise in cell count was attributed to "swarming" or a mechanical or chemical dispersion of the clumps. For all studies herein reported, except those using seed type A, every effort was made to use non-flocculated cells, and bacterial growth did not lag oxygen uptake. If flocculated seed was used by Lee and Oswald this could explain why the apparent plateau, which can be surmised from their data, comes slightly before the range of maximum population.

Based upon the results of the present investigation, it is possible to propose various mechanisms of causation for the plateau. The following possibilities seem prominent:

1) In a heterogeneous cell system, after reaching the stage of maximum growth (cell production or combination of cell production and storage), the predominating cells would begin to die or otherwise lose their turgor and release cellular components which may become food for other species of cells. Indeed, the bacterial cells themselves may become food for predator organisms. Time may be required before these secondary predominants can grow to such numbers that their growth is expressed in a measurable oxygen uptake. Such a sequential predominance of species may lead to two autocatalytic curves; the end of the first phase and beginning of the second may form the plateau.

2) After reaching maximum population, the cells may begin to die off and release cellular material. There may be no change in predominance, but because of the different structural configuration of the original substrate and the released cellular components, an acclimation period is required to synthesize the new enzymes needed to metabolize the new, and now exogenous, substrates. If enough cellular components are released, an after-growth may occur. On the other hand, the released components may not be of sufficient quantity to support much new growth, and the dieoff in total population continues. It is also possible that the released components may provide enough energy to maintain the viable integrity of the cells, and cell dieoff proceeds more slowly.

Theories I and II differ only in that the secondary stage of oxygen uptake is caused by a new predominance in the population in

Theory I, and by the same population with a required acclimation period in Theory II. The fact that the plateau is short in some cases and long in others can arise by variations in: a) time for acclimation, and b) time to gain enough population to be expressed in oxygen uptake.

3) After reaching maximum population density the cells do not die, in the sense that they can no longer respire, but may lose their ability to replicate. An acclimation period is needed before cellular components formerly synthesized in replication processes can be broken down to supply energy. Another possibility within the scope of Theory III is that an acclimation period is needed before the cells can use "storage products" for energy. In this case the length of the plateau, and indeed its existence in such systems, would depend upon the nature of the storage products and the induction time, if required, for producing the necessary catabolic enzymes. Reactions of this type might provide an explanation for the delayed onset of endogenous respiration suggested as possible cause for the plateau by Wilson and Harrison (8).

4) During rapid metabolism of some compounds in the first stage of oxygen uptake, the cells may release intermediates into the medium rather than channelling all such compounds into storage products. In such cases time may be required for induction of enzymes to initiate metabolism of the new exogenous compounds. Time for the induction of transport catalysts may also be required to bring these new components to existing enzyme systems within the cells. Since there is no longer enough substrate to support continued growth, the cells begin to die off.

It is entirely possible that all four theories contribute to causing the plateau. In addition, work reported from the authors' laboratory has shown that a diphasic oxygen uptake curve with the two phases separated by a pause or plateau can be generated due to the sequential removal of two exogenous substrates (19). Although this may be a valid cause for the existence of a plateau, it cannot be cited as the one or major general mechanism, since the plateau occurs with single exogenous carbon sources and at times also in endogenous systems which are devoid of exogenous carbon sources (washed seed blanks). However, this finding has important bearing on diphasic oxygen uptake in the BOD bottle since the release of cellular intermediates during metabolism of a single carbon source can cause the system to become a multi-carbon source. Also, it might be argued that Theory I is not generally applicable since the plateau has been found to occur using pure culture systems as well as heterogeneous populations. However, the major interest in the water pollution control field must lie in the area of heterogeneous populations, and although there may be several valid causes for the generation of a plateau, it is the one of most probable occurrence in the BOD bottle which will ultimately be the most important one. Thus Theory I must be given serious consideration. It is evident by the resultant formulation of four separate theories of causation that the mere measuring of viable cell population is not a sufficient basis for the formation of conclusive theory. However, this parameter was measured simply to form a logical basis for postulating theories of causation which provide avenues of investigation for deeper penetration into the problem. In this respect the parameter of choice was extremely valuable and has provided a basis for experimental investigations currently under way, to either prove or disprove these theories.

It may be discerned that although all four theories differ in essential components, there is one central likeness. The plateau is considered to be caused by a change from metabolism of the original exogenous carbon source to the metabolism of new carbon sources produced by the cells. Indeed, in heterogeneous populations the cells themselves may become a substrate. Work is continuing in this area in both high and low energy systems with the primary aim of seeking general or unifying theories of causation for phasic phenomena during the course of waste water purification.

SUMMARY AND CONCLUSIONS

1) The occurrence of the plateau in BOD exertion would appear to be independent of the type of seed employed. It is also exhibited over a wide range of substrates. It occurs in both single carbon source systems and systems containing a complex mixture of organic compounds. Within the usual limits of seed concentration employed in the BOD test, the existence of the plateau is not dependent upon seed concentration.

2) In general, under "normal" seeding conditions and substrate concentrations in the BOD bottle, the first phase of oxygen uptake corresponds to a period of rapid cellular multiplication, the plateau to the maximum range of population density, and the second stage of oxygen uptake to a decrease in the viable population.

3) The duration or severity of the plateau corresponds approximately to the length of the stationary phase in the region of maximum population density.

4) Based upon the above findings, four related theories explaining the existence of the plateau were formulated. These theories are similar in that the plateau is caused by a change from metabolism of the original exogenous carbon source to the metabolism of new carbon sources produced in the first stage of oxygen uptake. The plateau comes about because of either the need of an induction period before the new energy source(s) can be metabolized, or because of the time required for growth of secondary predominants to a density sufficient to exhibit a measurable oxygen uptake. It seems evident that there must be sequential metabolism of the primary and secondary substrates (whether the latter are the cells themselves or components excreted or stored by the cells) in order for a plateau to occur.

ACKNOWLEDGEMENTS

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PAPER 9

STUDIES ON THE CAUSATION OF PHASIC OXYGEN UPTAKE IN HIGH ENERGY SYSTEMS

By

M. N. Bhatla and A. F. Gaudy, Jr.

This paper was presented at the 19th Purdue Industrial Waste Conference, May, 1964, and it has been accepted for publication in the Journal Water Pollution Control Federation.

STUDIES ON THE CAUSATION OF PHASIC OXYGEN UPTAKE IN HIGH ENERGY SYSTEMS

By

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During the last decade the diphasic nature of the expression of carbonaceous BOD has been recognized. More recently it has been observed that a plateau in accumulated oxygen uptake can occur separating the two phases of oxygen utilization. It is this latter finding which has emphasized the need to re-evaluate present concepts of BOD kinetics. Many investigators agree that the first stage of O_2 uptake corresponds to assimilation of the exogenous carbon sources, and the second to endogenous respiration of the organisms which have grown up during the first phase. However, relatively little is known about the existence, the general occurrence, or the reason(s) for the pause or plateau which often occurs between the two phases.

Efforts to gain an insight into these aspects have precipitated a fairly large scale research effort in our laboratory. In general, two experimental approaches have been used in these investigations. First it was necessary to establish the range of conditions under which the plateau occurred and the generality of its occurrence. In accomplishment of this aim approximately fifty long-term BOD experiments

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were conducted in which the time course of BOD exertion and changes in the viable bacterial population were studied. Experiments were conducted in which the type and the concentration of seed material were varied. These experiments included investigations using various pure compounds as sole carbon sources, mixtures of pure compounds, and whole wastes. In the great majority of cases a plateau in BOD exertion was observed between two distinct phases of oxygen uptake. In general the first phase corresponded to a rapid growth of bacterial cells, the pause or plateau to the range of the maximum population, and the second phase or oxygen uptake to a decrease in viable count. These results established that a plateau could be observed under a wide variety of conditions, and analysis of the oxygen uptake and count data led to the formulation of theories of causation for the existence of the plateau. These results have been reported in a series of papers.(1)(2)(3)(4) The major emphasis in approach then shifted to studies into the causation for the plateau, and examination of the second stage of oxygen uptake in relation to the theories postulated as a result of our previous work.

A number of laboratories have been active in recent investigations of BOD kinetics, and it is appropriate to review this work as it bears upon reasons for the occurrence of the plateau. Busch(5) concluded that oxygen uptake which occurred after reaching the plateau was due to both endogenous respiration and oxygen utilization by predators, i.e., protozoa. He felt that the bacteria-protozoa relationship in the inoculum was a decisive factor in the clear expression of the plateau. However, Wilson and Harrison(6) obtained a two-phase oxygen up-take curve, i.e., two phases separated by a plateau, using a pure culture inoculum and phthalic acid as substrate. These workers related the magnitude of the secondary rise in oxygen utilization to the number of bacterial cells present in the system. They attributed this secondary rise in O_2 uptake to endogenous respiration, and stated that the plateau was probably due to the delayed onset of endogenous respiration. However, they offered no explanation as to why endogenous respiration should be so delayed. This work was accomplished in the Warburg apparatus using a fairly high concentration of phthalic acid. In our laboratory we have demonstrated the occurrence of the plateau, using a pure culture prototrophic strain of *Escherichia coli* in the BOD bottle.

Although the plateau has been shown both for certain pure culture strains and heterogeneous populations in dilute systems (BOD bottle), and in fairly high substrate systems (Warburg apparatus), it would appear from the recent report of McWhorter and Heukelekian(7) that substrate concentration can have an effect on the occurrence of the plateau. In Warburg studies using sewage seed with varying glucose concentrations, these workers observed the plateau at 100 and 500 mg/l but not at 1000 mg/l glucose. They offered no explanation for the occurrence of the plateau, but felt that their data did not agree with Busch's explanation since they reasoned that the disappearance of the plateau with increasing substrate concentration could not be attributed solely to changes in the predator-bacteria ratio.

Based on work in our laboratory, we have presented four related theories of causation of the plateau.(2) The work herein reported was concerned primarily with seeking a greater insight into these mechanisms of causation. All experiments herein reported were conducted with high energy systems; that is, using substrate concentrations much higher than those employed in the BOD test. Systems with high substrate concentrations were required for the study of some biochemical parameters which are difficult or impossible to evaluate in the BOD bottle. In the studies herein reported investigations were made on the effect of substrate concentration and initial solids concentration, and on the occurrence of the plateau using both sewage seed and acclimated seed. In addition, long term aeration studies were made to determine possible relationships between phases of oxygen uptake and substrate removal, intracellular protein content, and solids production. Efforts were also made to determine whether carbon sources other than the original carbon source were excreted into the medium during the plateau or the second stage of oxygen uptake.

MATERIALS AND METHODS

Settled sewage obtained from the municipal sewage treatment plant at Stillwater, Oklahoma, was used as seeding material. For some studies the sewage seed was used directly, while for others the sewage was used as an initial inoculum for obtaining an acclimated heterogeneous population. Acclimated populations were obtained by placing 2 ml of settled sewage in 60 ml of synthetic medium containing 5000 mg/l of glucose and the inorganic salt concentrations shown in Table 1. This system was aerated on a laboratory shaker at 100

Table 1

COMPOSITION OF SYNTHETIC WASTES

CONSTITUENTS	CONCENTRATIONS	
	I	II
Glucose	5000 mg/ℓ	100-1000 mg/ℓ
1 M Potassium Phosphate Buffer pH 7.0	30 mℓ/ℓ	10 mℓ/ℓ
$NaNO_3$	800 mg/ℓ	400 mg/ℓ
$MgSO_4 \cdot 7H_2O$	200 mg/ℓ	100 mg/ℓ
$FeCl_3 \cdot 6H_2O$	1 mg/ℓ	0.5 mg/ℓ
$MnSO_4 \cdot H_2O$	20 mg/ℓ	10 mg/ℓ
$CaCl_2$	15 mg/ℓ	7.5 mg/ℓ
Tap water	100 mℓ/ℓ	100 mℓ/ℓ
	MADE UP TO 1ℓ WITH DISTILLED WATER	

I. Synthetic waste used for growing acclimated seed.

II. Synthetic waste used in Warburg flasks.

strokes per minute, and temperature maintained at 25°C. After 24 hours 1 ml was transferred into fresh medium. Three serial transfers were made on successive days, and on the fourth day of operation cells were harvested near the end of the log growth phase (after approximately 18 hours of aeration) by centrifugation, washed twice in 0.05 M phosphate buffer, pH 7, and used for experimentation.

Oxygen uptake was measured using the Warburg respirometer (Gilson Medical Electronics). Substrate removal was measured using the standard COD technique,(8) and the anthrone analysis.(9)(10) Volatile acids were determined using the steam distillation and titration procedure described in Standard Methods.(8) For some studies protein was determined using the biuret method.(10) For experiments in which only small amounts of cells were available, protein was determined using a modification of the method of Lowry, et al,(11) employing Folin's phenol reagent. These modifications and verification of their applicability will be reported separately.

Since a variety of experiments were conducted, the general experimental protocol for each will be given as the results are presented.

RESULTS

Effect of Substrate Concentration

Since it had been reported previously by McWhorter and Heukelekian(7) that under experimental conditions similar to those herein employed, the plateau was discernible at 500 mg/l of glucose but not at 1000 mg/l, it was desirable to determine at what concentration the plateau could no longer be observed. These experiments were also important because it would be advantageous to work with a system which would yeild a plateau at the highest possible substrate concentration in order that sufficient material (substrates and cells) would be available for analysis. Accordingly, systems containing glucose concentrations from 100 to 1000 mg/l were examined for the existence of the plateau using both sewage seed and acclimated seed.

Figure 1 shows plots for accumulated oxygen uptake, using settled sewage seed. A distinct secondary stage of oxygen uptake was discernible at all concentrations employed. However, it is seen that as the concentration of glucose increased, the pause between the two phases of oxygen uptake became less promounced. The initiation of the secondary rise in O_2 uptake occurred at 36.5 to 47 percent of the theoretical oxygen demand. It is emphasized, however, that no correlation existed between the substrate concentration and either the time of occurrence of the second stage or the percentage of theoretical oxygen demand which had been exerted at the beginning of the second stage. The values shown in parentheses on each graph indicate percent of theoretical oxygen uptake expressed at the beginning of the second stage and at the termination of the experiment.

Results using acclimated seed are shown in Figure 2. These systems also show a distinct second phase at all substrate concentrations with the length of the plateau decreasing as substrate concentration was increased. The second stage was initiated at 39 to 45 percent of the theoretical oxygen demand. The lessening of the

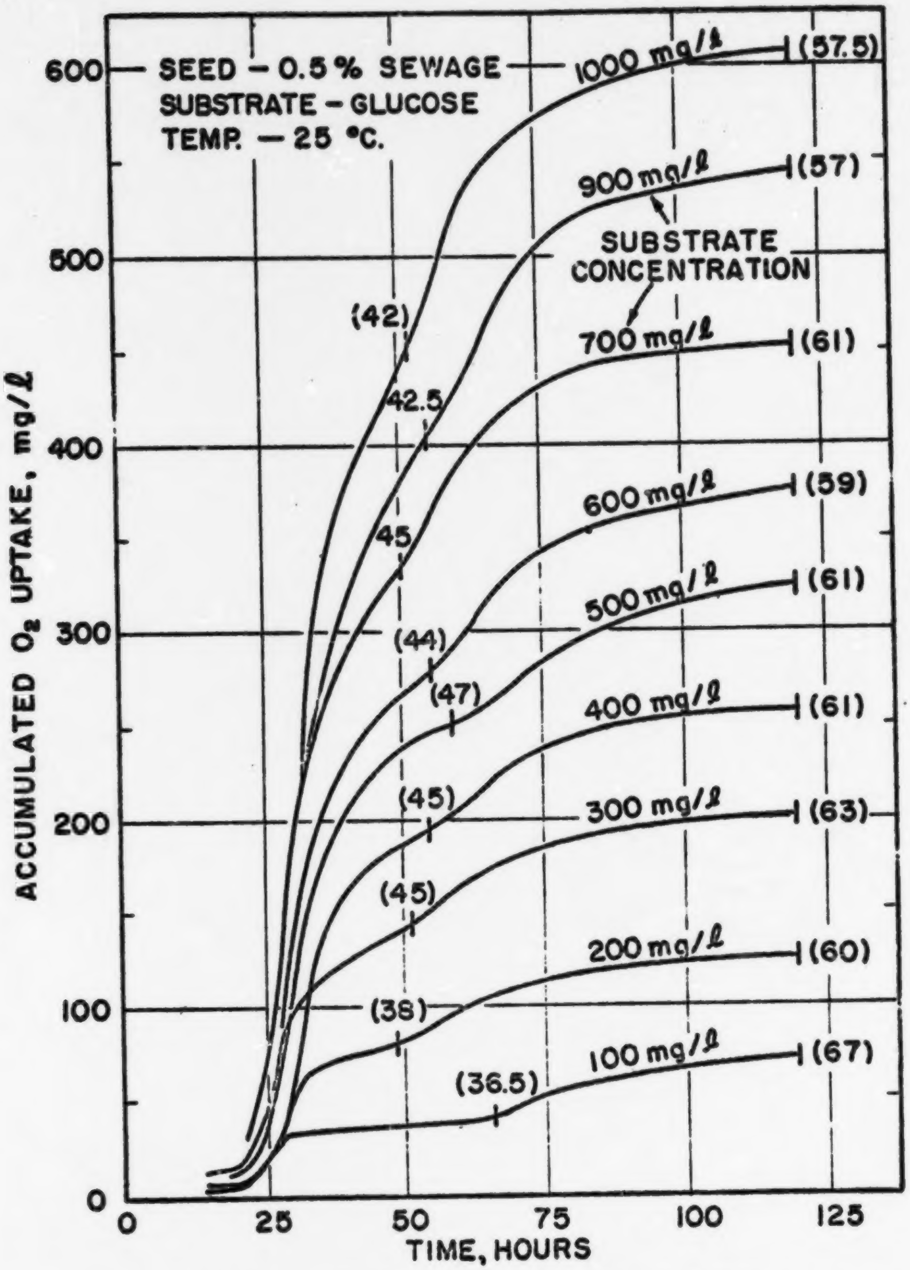

Fig.1—WARBURG STUDIES USING SEWAGE SEED. FIGURES ON CURVES ARE PERCENTAGES OF THEORETICAL OXYGEN DEMAND EXERTED AT THE TIME INDICATED.

initial lag and superimposability of the initial portions of the curves for these systems, as compared with those shown in Figure 1, is a result of the use of an acclimated seed and an initial solids concentration slightly higher than was used in the sewage seed experiments. It is also interesting to note that the initiation of the secondary rise for systems using acclimated seed is more constant with respect to time than that observed in Figure 1 using sewage seed. The results shown in these two figures indicate that the phasic oxygen uptake phenomenon occurs at all substrate concentrations from 100 through 1000 mg/l, but that at the higher substrate concentrations there is a tendency for the two phases to merge.

Effect of Initial Solids Concentration

Accumulated oxygen uptake curves using initial solids concentrations of 8, 25, 33, 65, 210, 250 and 500 mg/l, are shown in Figures 3 and 4. In these studies glucose concentration was maintained at 500 mg/l. An increase in initial solids concentration tended to shorten the lag or plateau between the two phases of oxygen uptake; however, a plateau was observed at all initial solids concentrations employed. It is also important to note that in these studies as with others in our laboratory, oxygen uptake in the endogenous flasks also exhibited a secondary rise.

Results thus far presented suggest that two phases of oxygen uptake exist, regardless of substrate concentration or initial solids concentration. However, the lag between the two phases is shortened by high substrate concentration or high initial solids concentration. While the substrate concentration had no significant effect on the time

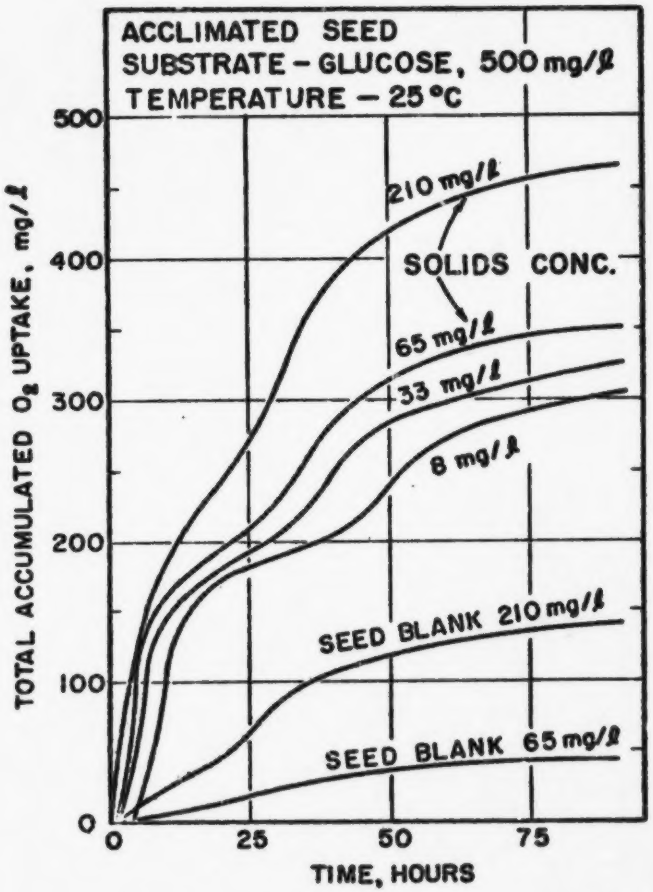

Fig. 3 - WARBURG STUDIES WITH VARYING CONCENTRATIONS OF INITIAL SOLIDS

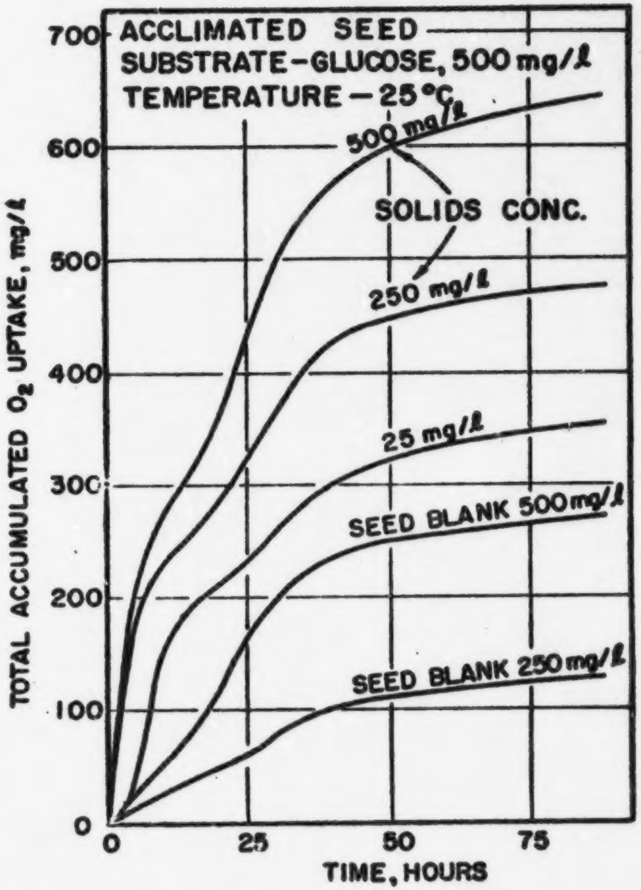

Fig. 4 – WARBURG STUDIES WITH VARYING CONCENTRATIONS OF INITIAL SOLIDS

of occurrence of the second phase, an increase in solids concentration appeared to hasten the occurrence of the secondary oxygen uptake phase.

Biochemical Response During Long Term Aeration Studies

In efforts to delineate further the mechanisms of causation for the plateau, long term Warburg studies were set up and, as the experiment progressed, flasks were removed for examination of internal system parameters. The results of three such experiments using both sewage seed and seed acclimated to glucose are shown in Figures 5, 6, and 7.

Figure 5 shows variations in COD, biological solids, oxygen uptake, and intracellular protein content throughout the experimental period. In this study 0.5 percent sewage seed was used. It is seen that there was a slight rise in the COD curve at approximately 80 hours. However, this occurred long after the plateau in oxygen uptake. From this result it appears that the plateau was in no way related to the excretion of cellular material responsive to the COD test. The plateau in oxygen uptake occurred at approximately 35 percent of the theoretical oxygen demand, and it is seen that the peak in the biological solids curve occurred slightly earlier than the plateau. The secondary stage of oxygen uptake was accompanied by a decrease in biological solids and a concomitant decline in cellular protein.

The results shown in Figure 6 were obtained using an initial inoculum of 1 percent sewage seed which had been filtered through Whatman No. 1 paper to reduce the predator population, as recommended

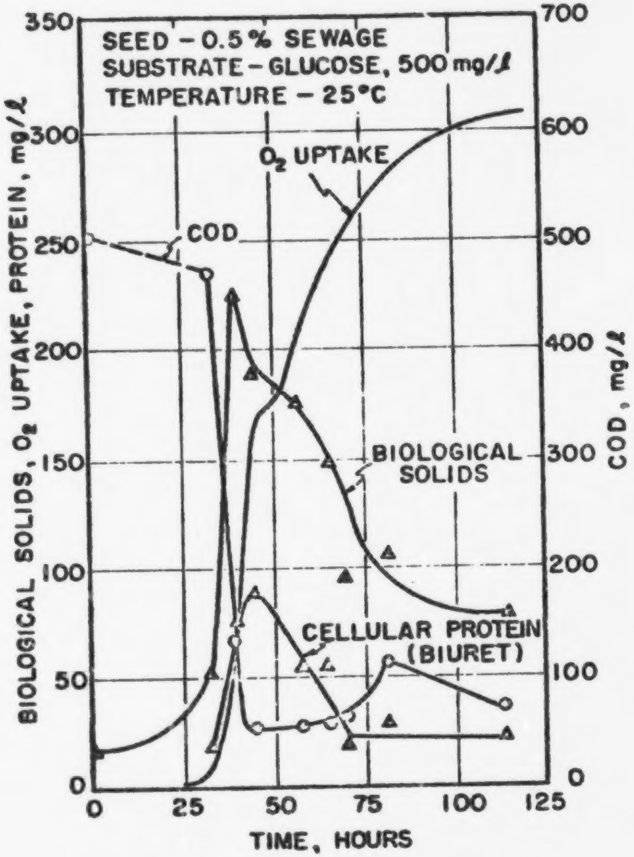

Fig. 5 -- LONG TERM AERATION STUDY USING SEWAGE SEED

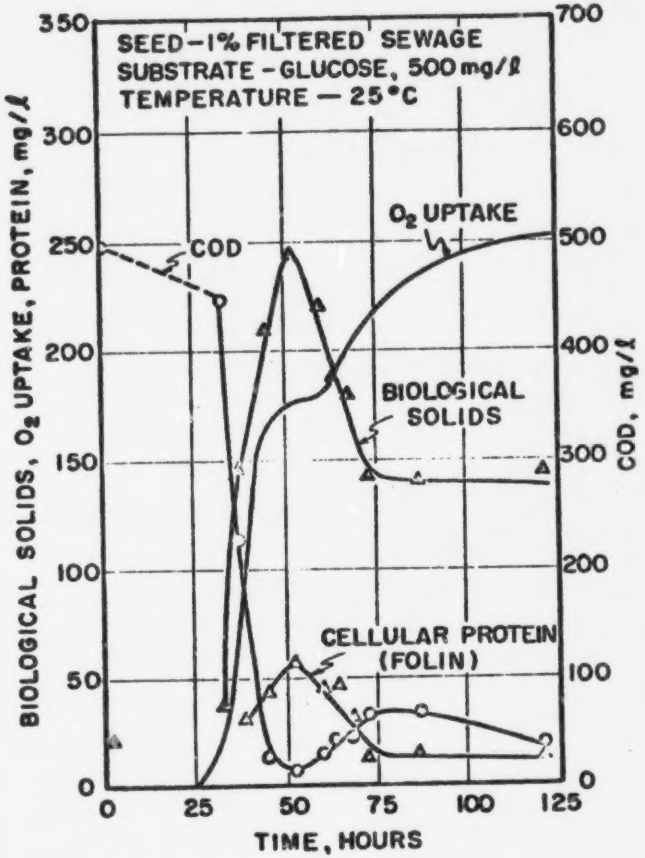

Fig. 6 - LONG TERM AERATION STUDY USING SEWAGE FILTERED THROUGH WHATMAN NO. 1 PAPER.

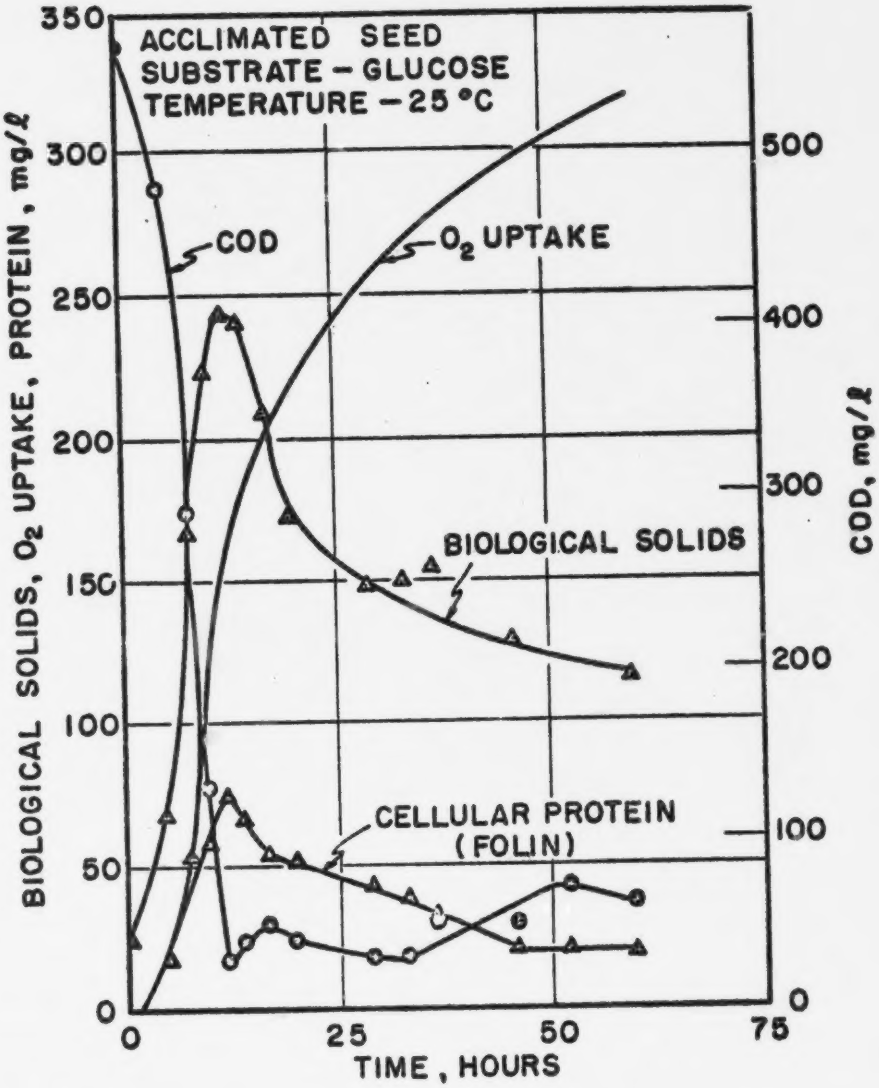

Fig. 7 - LONG TERM AERATION STUDY USING ACCLIMATED SEED

by Busch.(5) The COD removal pattern was essentially the same as that shown in Figure 5; a rise in COD in the latter portion of the experiment was also observed. It is of special interest to note that the 5-day oxygen consumption in the system using filtered sewage was 20 percent less than in the system using settled sewage seed, although the plateau appeared in both cases when approximately 35 percent of the theoretical oxygen demand had been exerted. The lower amount of oxygen used in the second stage of oxygen uptake is reflected in the lesser decline in biological solids shown in Figure 6 as compared to that in Figure 5.

For the results shown in Figure 7 an acclimated seed was employed. It is seen that in this experiment the COD removal pattern, solids production, and cell protein pattern are the same as those shown in the previous two figures. However, no pause or plateau in oxygen uptake was observed in the region of maximum solids production or at the point of substrate exhaustion.

From the results thus far presented it would appear that the plateau, when it occurs, is not associated with the release of intermediates or secondary substrates which are responsive to the COD test. This would indicate that if secondary substrates were used in the second stage of oxygen uptake and if the plateau were caused by the need for acclimation to such substrates, they would probably be intracellular carbon sources. Alternatively, the plateau could be caused by a secondary predominance, possibly involving predators.

There was a possibility that substrates not responsive to the COD test could be excreted into the medium. For example, certain of the volatile acids might not fully register as COD. Experiments similar to those previously presented but for which flasks were taken off for volatile acid analyses were included. The sample volume used in each analysis was from 70 to 90 ml, that is, the filtrate from two to three Warburg flasks. The results are shown in Figure 8. It is seen that a distinct plateau occurred, and that its initiation was correlated with the point of maximum removal of substrate. It is also seen that COD removal was paralleled by glucose removal as determined by the anthrone test. During the second stage of oxygen uptake there was a rise in the COD curve which was accompanied by a rise in the anthrone curve, indicating that much of the released COD was responsive to anthrone; that is, it was carbohydrate in nature. Very small amounts of volatile acids were present during the plateau and the second stage of oxygen uptake. From this experiment it would appear that valid conclusions concerning substrate concentrations before, during, and after the plateau, can be drawn from COD and anthrone data. It would appear that cellular components can be released during the second stage and, therefore, could contribute to the second stage of oxygen uptake.

Another experiment pertinent to this aspect was run in which two Warburg flasks were set up containing 500 mg/l of glucose synthetic waste seeded with 0.5 percent settled sewage. Oxygen uptake was followed in both flasks, and at the plateau (see arrow in Figure 9) the cell mass in flask B was centrifuged and the cells resuspended in 40 ml of inorganic buffer salts medium, identical in composition to that used in the synthetic waste, and again placed on the Warburg

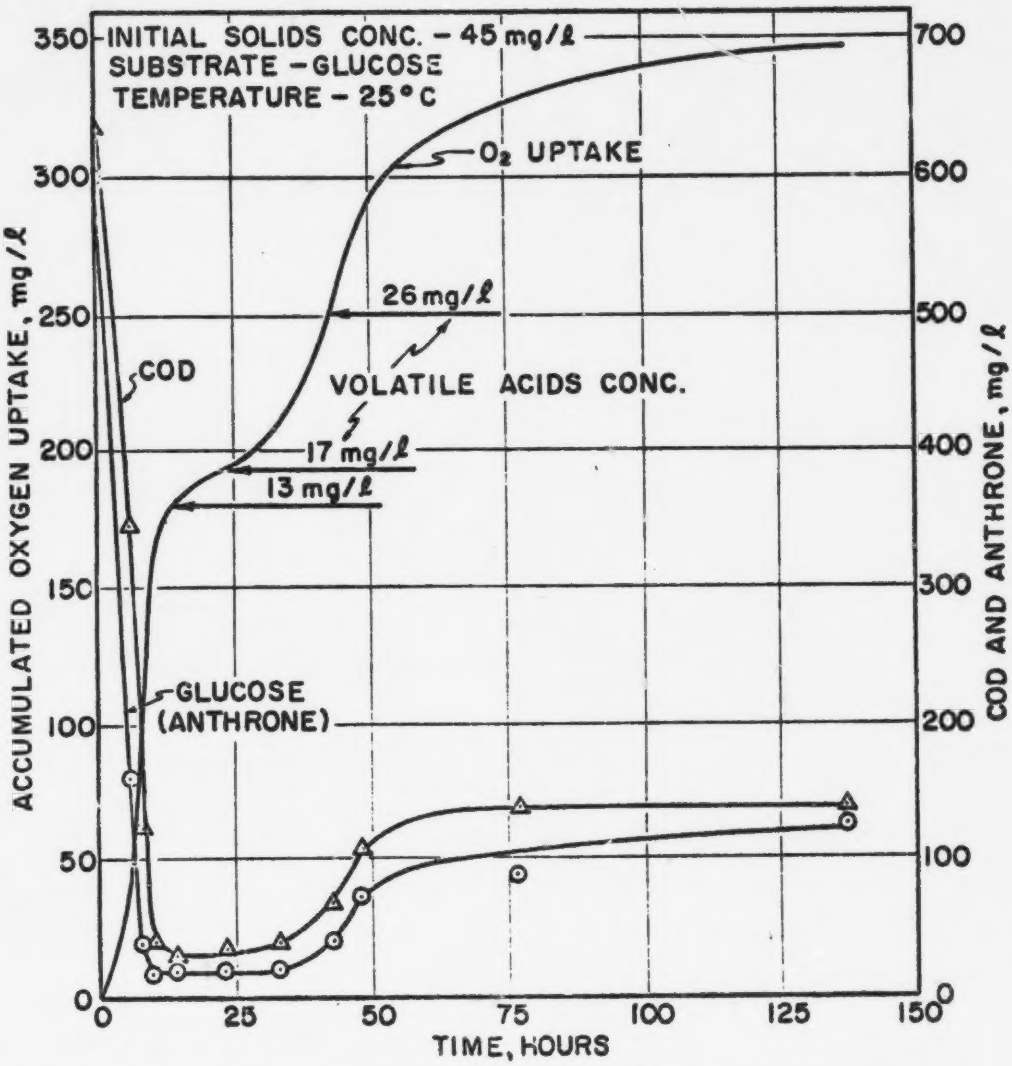

Fig. 8 - LONG TERM AERATION STUDY USING ACCLIMATED SEED.

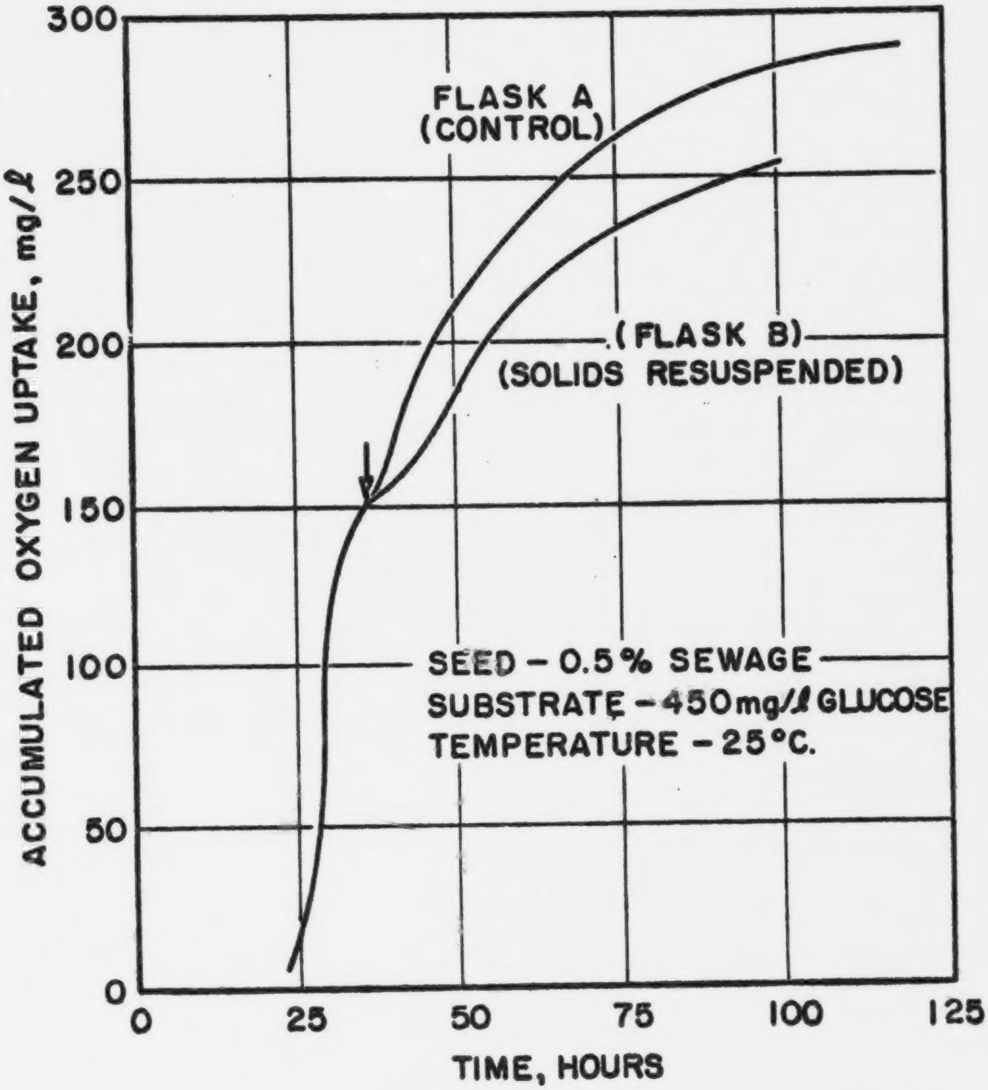

Fig. 9 – WARBURG STUDY USING SEWAGE SEED

apparatus. It is seen that the oxygen uptake in flask B was approximately 30 mg/l less than that in the control flask A. However, there was a considerable amount of oxygen used in flask B, indicating that the secondary stage of oxygen uptake observed in the control could have been due only in small measure to extracellular carbon sources.

DISCUSSION

The work herein presented offers a considerable insight into the delineation of basic mechanistic causes for the occurrence of the plateau in relation to the theories we have previously presented. One of the purposes for initiating these studies was to test these theories with the aim of eliminating some or possibly combining two or more to provide a sound generalized concept. While this work is continuing and encompassing aspects other than those dealt with here, the present work is valuable since it allows us to remove from serious consideration as a general cause for the plateau certain possibilities which logically could not have been eliminated without these investigations. Indeed, the theories we have eliminated as probable causes for the plateau in heterogeneous populations may still be invoked to explain the occurrence of a plateau in oxygen uptake in some systems. However, based upon our results, they cannot explain the plateaus observed in these experiments, and are therefore not considered to be general causative mechanisms in heterogeneous populations.

Briefly, the theories we have proposed are as follows:

1) After reaching the maximum population, some of the cells in heterogeneous populations begin to die off and release intracellular components which may become food for other organisms. Since the

latter organisms were not the predominating ones, time is required before they are present in sufficient numbers so that their metabolism of the released components is expressed in measurable oxygen uptake, hence, the appearance of a plateau or pause between the first and second stages of oxygen utilization. Alternatively, the cells themselves may become a substrate for predators.

2) After reaching maximum population some of the predominating organisms die and release intracellular components. There may be no change in the predominating species, but an acclimation period may be required before the living or intact cells can metabolize the released components.

3) After reaching maximum population the cells may not die off, but may lose their ability to replicate. An acclimation period may be required before the cells can metabolize certain cellular components which were synthesized in the replicating process (e.g., excess protein or nucleic acid); that is, an acclimation period may be required before certain cellular components can be endogenously metabolized.

4) During the rapid metabolism which takes place in the first stage, the cells may release intermediates into the medium. An acclimation period may then be required before these new exogenous compounds can be metabolized. Also, an induction period may be needed to produce catalysts to transport these compounds back into the cell.

These are theories which could explain the existence of a plateau in oxygen uptake in systems employing single or multicomponent carbon sources. In multicomponent media, sequential utilization of exogenous substrates can also cause diphasic oxygen uptake as we have previously shown in high energy systems.(9) All four theories could apply to heterogeneous populations, but theory 1 obviously cannot be invoked to explain plateaus observed for pure culture systems.

It is quite impossible to eliminate, unequivocally, any of these theories, but based upon the parameters measured for the systems herein studied it is possible to conclude that the plateaus observed were not caused by the release of cellular intermediates during the first stage (theory 4). This is borne out by the results shown in Figure 8, where it was seen that there was no exogenous substrate present in the medium at the time of occurrence of the plateau.

COD data for the experiments shown in Figures 5, 6, 7 and 8 indicate release of soluble organic material into the medium at various times during the second stage, but there is no indication in any experiment of such a release at a time which could affect the occurrence of a plateau as postulated in theory 2. However, the amount of second stage oxygen uptake attributable to the release of soluble organic material cannot be determined from present data since only the unused material is measured by the COD test. There is no reason to assume that only nonutilizable material would be released. The results shown in Figure 9 also tend to lessen the general applicability of theory 2, since it was seen that over 75 percent of the oxygen used in the second stage could be attributed specifically to metabolic activities of the particulate fraction of the system, that is, the biological population itself. This surmise is also substantiated by the fact that washed cell suspensions used for endogenous blanks have generally been observed to yield a plateau. Work is currently under way pertaining to further delineation of causation, stressing the possible roles of the mechanisms included in theories 1 and 3.

It was seen from Figures 1 and 2 that there was no distinct or critical substrate concentration at which the plateau could not be observed. We did not find the absence of a plateau at the 1000 mg/l substrate level, as did McWhorter and Heukelekian. The data do, however, indicate that the phases gradually merge and that the plateau tends to become indistinct and may finally disappear as the total energy of the system increases. The energy of the system may be increased either by adding a higher amount of substrate or by starting with a higher initial inoculum of cells. It is important to note, however, that in low energy systems (BOD bottle) an increase in initial seed did not cause the plateau to disappear for any practical range of seed concentration.(4)

It would appear that the merging of the two phases is probably associated with a higher endogenous respiration in the high substrate or the high initial solids systems. The term "endogenous respiration" is a rather nebulous one, since it means different things to different investigators. In a strict microbiological sense it has always meant the utilization for respiratory activity of internal carbon sources, regardless of whether an exogenous carbon source is present. In the water pollution control field it is usually understood to indicate the respiration of intracellular materials after the exogenous carbon source has been assimilated. This latter meaning is herein employed. In the high substrate systems more cells are produced; therefore there are more cells to undergo endogenous respiration. In the high solids systems large numbers of cells are placed in the medium by the experimenter but, in either case, there are more cells participating in the so-called endogenous respiration phase.

The definition of "endogenous respiration" which we normally use in the pollution control field becomes more complicated when we consider the role of predator organisms in a heterogeneous population since, in order to contribute significantly to oxygen uptake, they must be metabolizing bacterial cells which are for them an exogenous substrate. The possible role that predator organisms may play in generating the second stage of O_2 uptake and in causing the plateau has been the subject of some disagreement in the field. Certain aspects of their role have been postulated by Busch, but without experimental proof.(5) On the other hand, the results of Wilson and Harrison(6) and of McWhorter and Heukelekian(7) have led these researchers to present objections to the views of Busch. The pure culture studies of Wilson and Harrison in high substrate concentrations and our own in low energy systems (the BOD bottle), showing a plateau, leave little doubt that the predator-bacteria ratio cannot be cited as the only cause for the observance of a plateau. However, it is important to note here that current research in our laboratory conducted in investigation of theory 1 is providing fairly strong evidence that in many systems the predator-bacteria ratio can determine whether a plateau will be observed. These studies will be the subject of a future report. As the work proceeds, we are finding that there may be several valid causes for the production of a plateau in oxygen uptake.

In summary, it would appear that the release of cellular components due to lysis of a portion of the bacterial population after reaching the maximum attainable numbers and the need for an acclimation period before metabolism of these released components is not a general cause for the plateau. Also, it would appear that the release of intermediate or end products during the metabolism of the original exogenous substrate is not a general cause for the plateau. While these theories cannot be unequivocally eliminated for all systems, the results herein presented indicate that they do not provide potentially fruitful areas for further investigations into general causation when considering heterogeneous populations. Also, from these studies it was seen that while an increase in initial substrate concentration and in cell concentration exhibited a compressing effect on the plateau, it was observed at substrate concentrations varying from 100 to 1000 mg/l and in systems employing up to 500 mg/l of initial biological solids. It would therefore appear that neither substrate nor solids concentration, *per se*, plays a decisive role in the observance of a plateau. These results also emphasize (as seen by comparison of Figures 5 and 6 with Figure 7) that while such parameters as COD removal, biological solids and protein can show similar patterns, the oxygen utilization patterns can be drastically different. It would therefore appear unwise at this time to attempt to use the plateau as an indicator of such parameters in either research or analytical methodology for assessing pollutional strength of waste waters.

ACKNOWLEDGEMENT

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PAPER 10

STUDIES ON THE PLATEAU IN OXYGEN UPTAKE DURING EXERTION OF BIOCHEMICAL OXYGEN DEMAND BY PURE CULTURES OF BACTERIA

By

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STUDIES ON THE PLATEAU IN OXYGEN UPTAKE DURING EXERTION OF BIOCHEMICAL OXYGEN DEMAND BY PURE CULTURES OF BACTERIA

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SUMMARY

Evidence for generation of the plateau in oxygen uptake during exertion of carbonaceous BOD by pure cultures was sought by making long term Warburg studies using glucose and phthalic acid as substrates. One organism, tentatively identified as *Escherichia intermedia*, of the 57 tested, exhibited a plateau. Detailed studies on this organism indicated that generation of the plateau depended upon the type of substrate and to some extent upon the initial cell concentration employed, but not upon substrate concentration. Oxygen utilization during the second stage was accompanied by metabolism of secondary extracellular carbon source(s) produced by the cells during metabolism of the original extracellular carbon source (glucose) in the first phase of O_2 uptake. The secondary substrate was registered as volatile acids. Selective inhibition of protein synthesis at various times during oxygen uptake led to the conclusion that the plateau occurred because of the need for an induction period before the secondary substrate could be metabolized. Using this organism, it was also found that a plateau could be generated during endogenous respiration. This secondary O_2 utilization was accompanied by uptake of organic materials excreted into the medium by the cells during metabolism of endogenous carbon sources.

INTRODUCTION

The biochemical oxygen demand (BOD) test is extensively used for assessing organic pollution. The earliest observations on the kinetics of oxygen consumption in municipal waste samples resulted

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This work represents a portion of the research accomplished in partial fulfillment of the requirements for the Ph.D. degree by M. N. Bhatla.

in the general adoption of the first order decreasing reaction rate concept for exertion of carbonaceous BOD. Due largely to the work of Hoover, et al.(1) this concept has been, in general, replaced by a two-phase reaction concept, the first phase being one of rapid oxygen consumption attributable to cell multiplication and assimilation of exogenous substrate, while the second phase of oxygen uptake is attributed primarily to endogenous respiration of the microbial population. In addition, work by Busch on this respiration test has shown that carbonaceous BOD exertion can take place in two phases separated by a period of low oxygen absorption commonly known as a plateau(2). It is the latter type of oxygen absorption kinetics which has occasioned studies in the authors' laboratory for the purpose of establishing and verifying mechanisms of causation for this type of kinetic expression.

Previous investigations(3)(4) using dilute systems (BOD studies) established with reasonable certainty the substrate independence of the occurrence of a diphasic oxygen utilization curve containing a plateau. In other work the authors(5) have shown that this type of oxygen uptake curve may also be observed in high energy systems (Warburg studies) and that there appears to be no essential difference in high and low energy systems so far as the biochemical mechanisms causing the plateau are concerned. The types of organisms present in the BOD bottle can be divided into two major categories, i.e., bacteria and predator organisms (mostly protozoa). The need for considering other forms such as algae, etc., is obviated due to the limitations imposed by the BOD test conditions. The role of predators, i.e., protozoa, in the exertion of BOD will be reported elsewhere; the present studies deal with the occurrence and mechanism of causation of plateaus in systems containing only bacteria.

Very little information is available regarding the diphasic expression of oxygen uptake with purely bacterial systems; however, it is important to note that a plateau can be generated in such systems. The authors have observed two-phase oxygen absorption, with the two phases separated by a plateau, in low energy systems using a prototrophic strain of Escherichia coli and glucose as the sole carbon source(3). Another study showing bimodal oxygen uptake has been reported by Wilson and Harrison using a pure culture inoculum in phthalic acid(6). This study was conducted at fairly high substrate concentrations in the Warburg respirometer. The pure culture (isolated from activated sludge) in this case was not identified, but the authors felt that it belonged to the genus Pseudomonas(7). It was suggested that the second stage of oxygen uptake was due to late initiation of endogenous respiration; however, reasons for such a delay were not offered. Halvorson(8) observed two-stage oxygen absorption separated by a period of low oxygen uptake with Bacillus cereus var. terminalis. Cells were grown on glucose-yeast extract-mineral medium. While the first stage of O_2 uptake was due to the vegetative growth of the cells, the initiation of the second stage of oxygen uptake was concomitant with sporogenesis. Later investigation of the same system showed accumulation of pyruvic and acetic acids in the medium during the vegetative growth of cells(9). It was proposed that the transition phase in oxygen uptake was due to the induction of acetate-oxidizing enzyme systems. More recently it has been shown that B. cereus strain 1 does not possess the enzyme system responsible for the terminal oxidation of acetate to CO_2 in the vegetative stage(10). Marion and Malaney(11), in Warburg studies on oxidation of aliphatic compounds by Alcaligenes faecalis, observed a plateau in oxygen uptake for the endogenous seed flasks as well as for flasks containing substrate and growth medium. They suggested that this phenomenon was similar to a diauxic effect caused by acclimation to two successive intracellular substrates. The authors have also noted diphasic oxygen uptake in endogenous systems(3).

Diphasic oxygen uptake has thus been observed with several different bacterial species using different substrates. The purpose of the present studies was to investigate the extent and the nature of diphasic oxygen uptake due solely to bacterial metabolism. Cultures were isolated from various sources, and oxygen uptake and other parameters were studied using a sole source of carbon. These studies bear upon general theories of causation for the plateau previously given by the authors(3).

MATERIALS AND METHODS

Sources of Test Organisms

(1) Twenty-one pure cultures of bacteria capable of growing in minimal medium using a sole carbon source were obtained from the microbiology department at Oklahoma State University, Stillwater. These included nine strains of *Escherichia coli*, eight species of *Pseudomonas*, *Serratia marcescens*, *Micrococcus luteus*, and two strains of *Aerobacter aerogenes*.

(2) Five pure cultures were isolated from suspensions of mixed populations of sewage origin which exhibited diphasic O_2 uptake (including a plateau) in Warburg experiments.

(3) Other pure cultures were isolated from laboratory batch activated sludge units initiated with seed from the primary clarifier effluent of the municipal sewage treatment plant, Stillwater, Oklahoma. The units were fed daily with 1000 mg/l glucose or phthalic acid using a basal medium described below. The stock phthalic acid solution was adjusted to pH 7 with 20 per cent KOH before use. The cells were harvested after two to three days' growth. Nutrient agar (Difco) was used for isolating and storing cultures. Fourteen cultures were isolated from the phthalic acid unit, and seventeen from the glucose unit.

Basal Medium

Quantities of constituents used per liter of distilled water are as follows: $(NH_4)_2SO_4$, 1.0 gm; $MgSO_4.7H_2O$, 0.2 gm, $FeCl_3.6H_2O$, 0.001 gm; $MnSO_4.H_2O$, 0.02 gm; $CaCl_2$, 0.015 gm; 1.0 M potassium phosphate buffer, pH 7.0, 30 ml; tap water, 100 ml. Carbon sources were added as indicated below.

Analytical Techniques

Oxygen uptake was measured on a Warburg respirometer (Gilson Medical Electronics, Middleton, Wis.) at 100 oscillations/min and 25°C. Biological solids were determined by the membrane filter technique(12) (Millipore Filter Corp., Bedford, Mass.; HA, 0.45 μ). The total organic material and glucose remaining in the membrane filtrate were determined by the COD test(12) and the anthrone method(13), respectively. Protein and carbohydrate contents of the biomass were determined by the Lowry(14) and anthrone(13) methods, respectively. Volatile acids were determined by steam distillation(12).

Examination of Cultures for Plateau Production

The pure culture inocula were prepared for oxygen uptake studies, using the basal medium and 3000 mg/l of substrate, by four daily transplants of small inocula as previously described(15). After the fourth successive transfer, a small amount of cells (1% inoculum) was placed in fresh basal medium containing 500 mg/l of substrate (glucose or phthalic acid). Forty ml of each cell suspension were placed in a Warburg flask for measurement of O_2 uptake.

Effect of Cell and Substrate Concentration

A series of Warburg flasks was set up with various concentrations of glucose (100-900 mg/l) in basal medium and inoculated with 0.4 ml of a culture of glucose-grown pure culture No. 13. In other studies all flasks contained 500 mg/l glucose, and initial cell concentration was varied. The cell suspensions used in these studies were obtained as follows: Cells growing on glucose were harvested, washed twice in 0.05 M potassium phosphate buffer pH 7, and resuspended in basal medium. Portions of this suspension were appropriately diluted and added to the prepared medium to achieve the desired cell concentrations. Oxygen uptake in endogenous controls was also recorded.

Study of Biochemical Parameters

The basal medium was inoculated with a suspension of washed pure culture No. 13 and glucose stock solution was added in appropriate amounts to attain desired concentrations. Forty ml samples were then placed in Warburg flasks, and the course of O_2 uptake was followed. At various times flasks were removed and 25 ml of flask contents were passed through a membrane filter. The filtrate was used for the COD and anthrone determinations. An additional 10 ml of mixed liquor were filtered, and the cells were suspended in 3 ml of distilled water for carbohydrate and protein analysis. In cases where volatile acids were also determined, two to three flasks were removed and a sample volume of 60-88 ml of filtrate was used for volatile acids determination by steam distillation.

Endogenous Metabolism

Cells were harvested and washed in phosphate buffer, pH 7. They were resuspended in basal medium to yield the desired cell concentration. The experimental protocol was similar to that employed in the study of biological parameters, except that substrate was not added to any system.

Inhibition of Protein Synthesis

Warburg vessels equipped with sidearms were prepared using a small initial inoculum of washed cells and 500 mg/l of glucose. One ml of 2.0 mg/ml solution of chloramphenicol was placed in the sidearm and was tipped into the medium at the desired time. Control flasks which contained no chloramphenicol were also prepared.

RESULTS

Accumulated oxygen uptake data were obtained in 120-hour Warburg studies for each of the 57 pure cultures described above. Due to the general similarity in oxygen utilization patterns, oxygen uptake curves for only seven pure cultures isolated from the glucose unit are shown in Figure 1. The symbols on the curves are for identification purposes only, and do not represent plotted O_2 uptake values. Manometers were read at least each half hour during the early portion of each run and in the vicinity of the break in the curve, and at longer intervals after the first 75 hours of incubation. It is seen that in general the curves consisted of a phase of rapid oxygen uptake followed by a phase of slow uptake, and no plateau was observed. In all of these studies only one pure culture (No. 13 from the glucose unit) exhibited diphasic oxygen uptake, with the two phases separated by a plateau. This organism was tentatively identified as Escherichia intermedia on the basis of the following characteristics: The organism was a gram-negative rod, motile with peritrichous flagella. It fermented glucose, lactose, and sucrose with the production of acid and gas, and used citrate as a sole carbon source. Nitrate was reduced to nitrite; indole was produced in tryptone broth. Both starch and gelatin were hydrolyzed. The Voges-Proskauer test was negative, the methyl red test positive. Colonies on EMB agar had a metallic sheen. Hydrogen sulfide was not

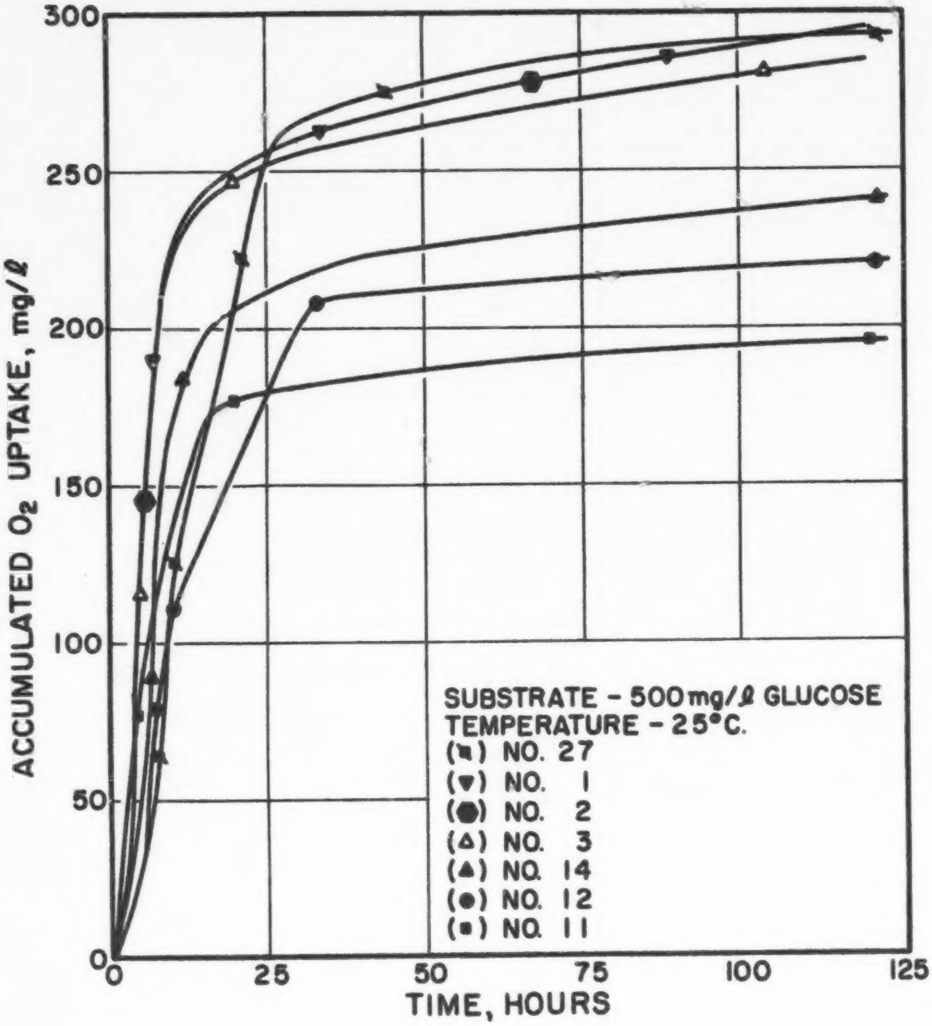

Fig. 1 - WARBURG STUDIES OF PURE CULTURES ISOLATED FROM A GLUCOSE-FED ACTIVATED SLUDGE UNIT.

produced. All of these characteristics, except gelatin hydrolysis, are consistent with the identification of the organism as *Escherichia intermedia*.

The test organism (pure culture No. 13) was studied in detail under different conditions in attempts to determine the cause for occurrence of the plateau. The constancy of its occurrence was examined under three conditions: 1) different substrate concentrations, 2) different amounts of initial cell inoculum, and 3) different types of substrates.

Effect of Substrate Concentration

Glucose was used as a substrate in these studies. Figure 2 shows plots for oxygen uptake using a small inoculum of culture No. 13 which had been previously grown on glucose. A distinct secondary rise in oxygen uptake was discernible at all concentrations of glucose employed; however, it is seen that the period of low oxygen uptake between the two phases was extended with increasing glucose concentrations. Nevertheless, the occurrence of the diphasic oxygen uptake was observed to be independent of the substrate concentration. The magnitude of oxygen consumed before the beginning of the second stage of oxygen uptake varied, increasing from 23.5 to 34.4 per cent of the theoretical oxygen demand as the glucose concentration increased; however, the percentage of the theoretical oxygen demand exerted at the end of 100 hours was essentially the same at all substrate concentrations.

Effect of Initial Cell Concentration

Oxygen uptake curves for systems using 500 mg/l glucose and initial cell concentrations of 13, 128, 256, 640, and 1280 mg/l of culture No. 13 are shown in Figure 3. While the system containing 13 mg/l initial cell concentration yielded two phases of O_2 uptake separated by a plateau, the 128 mg/l cell concentration did not exhibit a plateau. However, a plateau followed by a distinct second stage of oxygen utilization reappeared at 256, 640, and 1280 mg/l initial cell concentrations. Also it is seen that secondary oxygen demand in these systems (256, 640, and 1280) was proportional to the initial cells present. It is interesting to note that the endogenous controls for these cell concentrations (i.e., 256, 640, and 1280 mg/l) showed a concomitant secondary rise in oxygen uptake.

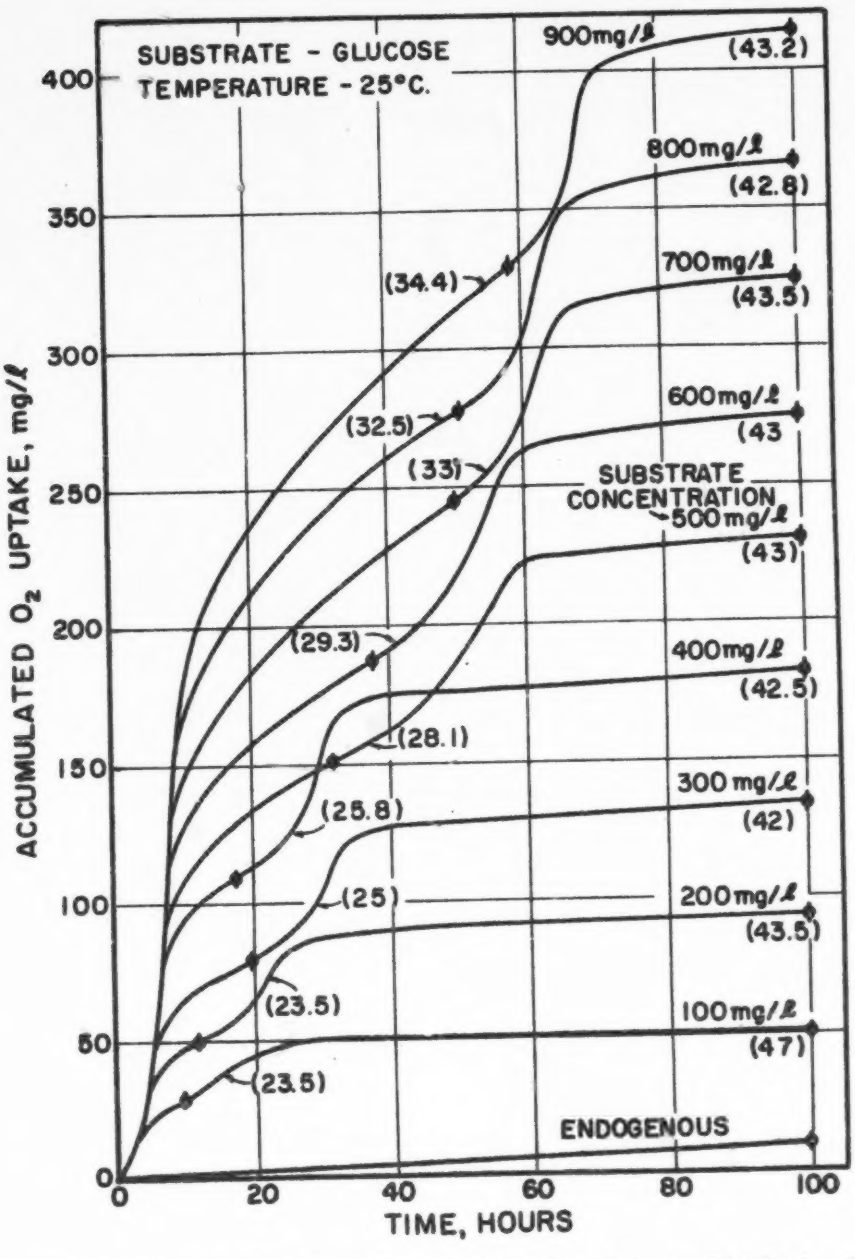

Fig. 2 - WARBURG STUDIES USING A SMALL INOCULUM OF CULTURE NO. 13 ACCLIMATED TO GLUCOSE. (Figures in parentheses are percent theoretical oxygen demand of the substrate exerted at the time indicated.)

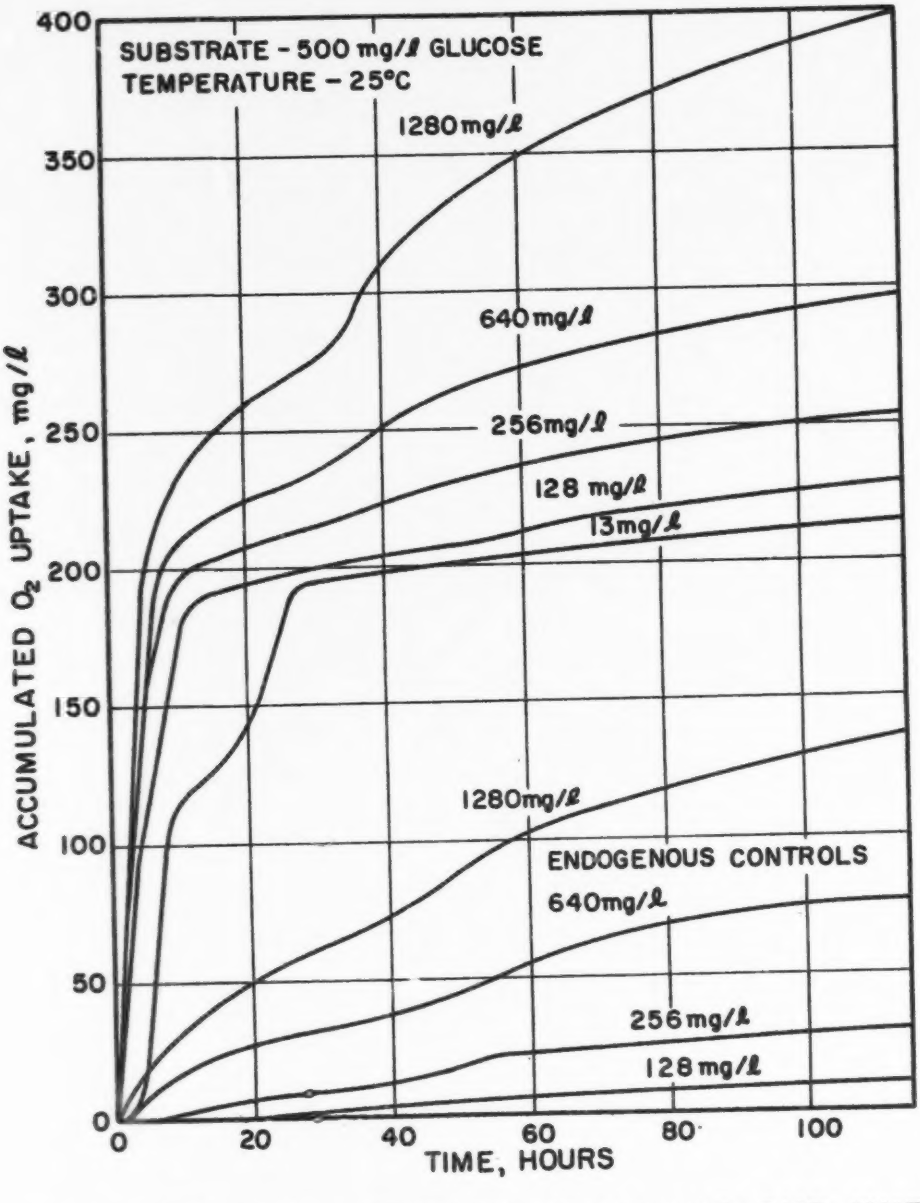

Fig. 3 - WARBURG STUDIES USING VARIOUS INITIAL SOLIDS CONCENTRATIONS OF CULTURE NO. 13. (Figures on curves are initial solids concentrations)

Moreover, the second stage of oxygen utilization in these systems (both endogenous and fed) occurred simultaneously and was delayed as compared to that in the lowest initial solids concentration (i.e., 13 mg/l). This same variation in the pattern of oxygen utilization with different cell concentrations was obtained on repetition of the experiment.

Effect of Type of Substrate

Oxygen uptake curves obtained using a variety of substrates inoculated with small amounts of acclimated cells (culture No. 13) are shown in Figure 4. A diphasic oxygen utilization curve was recorded for both d-sorbitol and D-(-)-ribose; however, the separation of the two phases for ribose was not pronounced. There was a rapid phase of oxygen uptake followed by a slow phase for the acetate and glutamate systems, and no secondary rise in oxygen absorption was discernible. A rather delayed secondary rise in oxygen utilization was observed for nutrient broth (Difco).

Another study similar to the one reported above was carried out using an initial inoculum acclimated to glucose. Apart from differences in the lag periods, results similar to those shown in Figure 4 were obtained for all substrates except for nutrient broth, for which no secondary rise was observed. In addition, oxygen uptake was studied with a glucose-acclimated inoculum in sterile sewage. No plateau or second stage of oxygen uptake was observed. In summary, except for the nutrient broth system, the initial acclimation of cells to the substrates did not significantly affect the O_2 utilization pattern.

Study of Biochemical Parameters

Figure 5 shows variations in biochemical parameters for a three-day Warburg experiment. It is seen that the time of glucose removal, as measured by the anthrone test, corresponded to the break in the oxygen uptake curve and that the COD curve is essentially a "mirror image" of the oxygen utilization pattern. At the time all glucose was removed from the system, only half of the COD had been removed. Volatile acids accumulated in the medium, reaching a maximum concentration of 130 mg/l (measured as acetic acid) near the beginning of the second stage of oxygen uptake. This was removed during the second

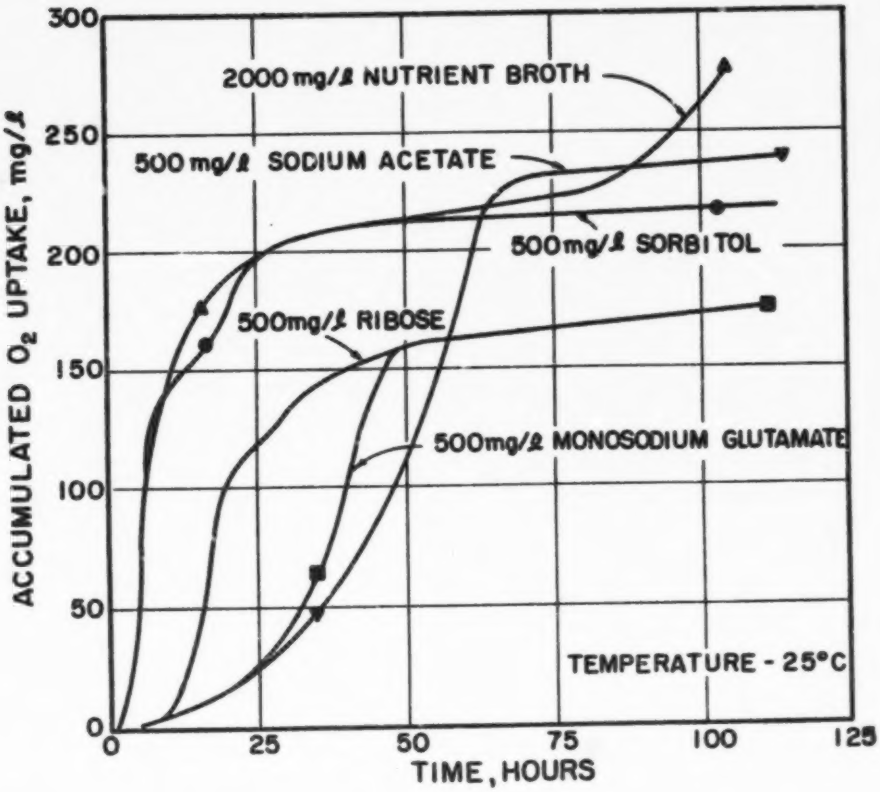

Fig. 4 - WARBURG STUDIES ON VARIOUS COMPOUNDS USING SMALL INOCULUM OF CULTURE NO. 13 ACCLIMATED TO THE CARBON SOURCE.

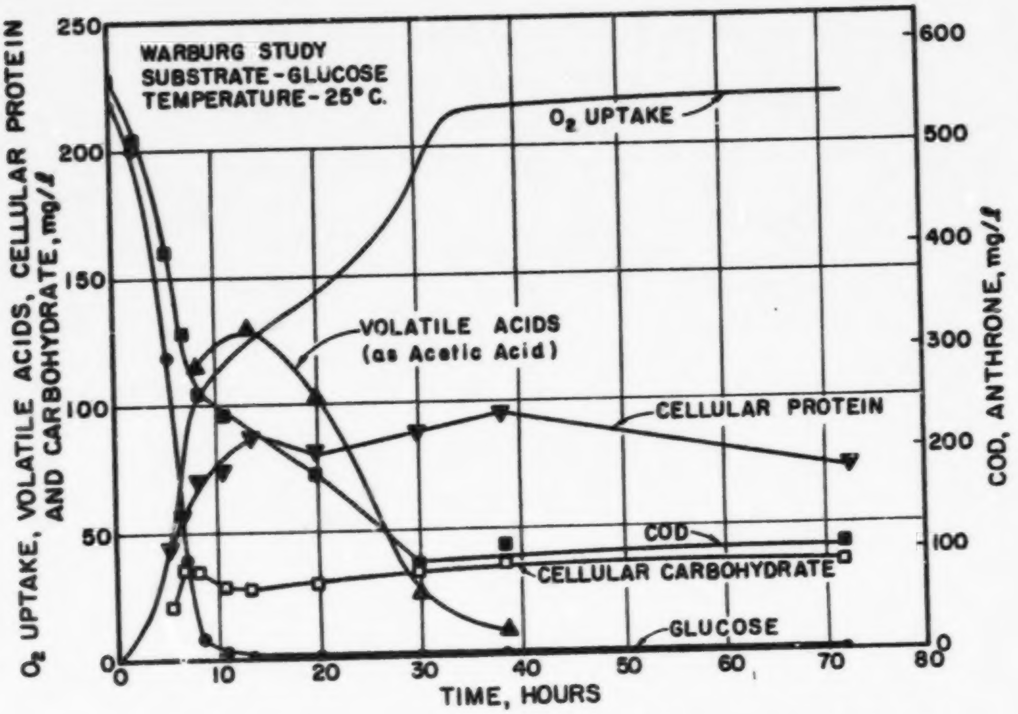

Fig. 5 - GLUCOSE METABOLISM BY CULTURE NO. 13.

stage of oxygen utilization. Residual COD of approximately 100 mg/l persisted in the medium. Cellular protein content reached a peak corresponding to the point of maximum volatile acids in the medium, and attained another peak at the end of the second stage of O_2 uptake. A very small drop in cellular protein was noticed toward the end of the experiment. Cellular carbohydrate content reached a maximum much earlier, and a slight rise in carbohydrate content was also observed during the second stage of oxygen utilization.

Inhibition of Protein Synthesis

An inhibitor of protein synthesis, chloramphenicol(16), was employed to examine the possible involvement of a readjustment of the enzyme system(s) prior to the second phase of oxygen uptake. It was intended to block protein synthesis at various times relative to glucose removal by the addition of chloramphenicol and to study its effect on oxygen uptake.

The results of such an experiment are shown in Figure 6. Chloramphenicol was tipped from the sidearms into the main reaction chambers of Warburg flasks A, B, and C (see arrows, Figure 6) to give a final concentration of 50 µg/ml in each flask. Figure 6 shows modifications in the oxygen uptake pattern in flasks A, B, and C, as compared to the control flask. Glucose was removed by the 9th hour. The oxygen utilization in flask A, in which the chloramphenicol was added at 6.5 hours decreased sharply, as compared to the control system, after the time of glucose removal. Subsequently very little oxygen (approximately 10 mg/l) was utilized by the cells in flask A. The oxygen uptake in flask B, in which the inhibitor was added 1.5 hours after the point of glucose exhaustion, also decreased as compared to the control. In flask B only half as much oxygen was used as in the control from the point of addition until the end of the experiment. The addition of chloramphenicol to flask C, when the secondary rise in oxygen uptake was well underway, caused the oxygen uptake in flask C to lag that in the control. However, at the end of the experiment 14 mg/l more oxygen had been consumed in flask C than in the control.

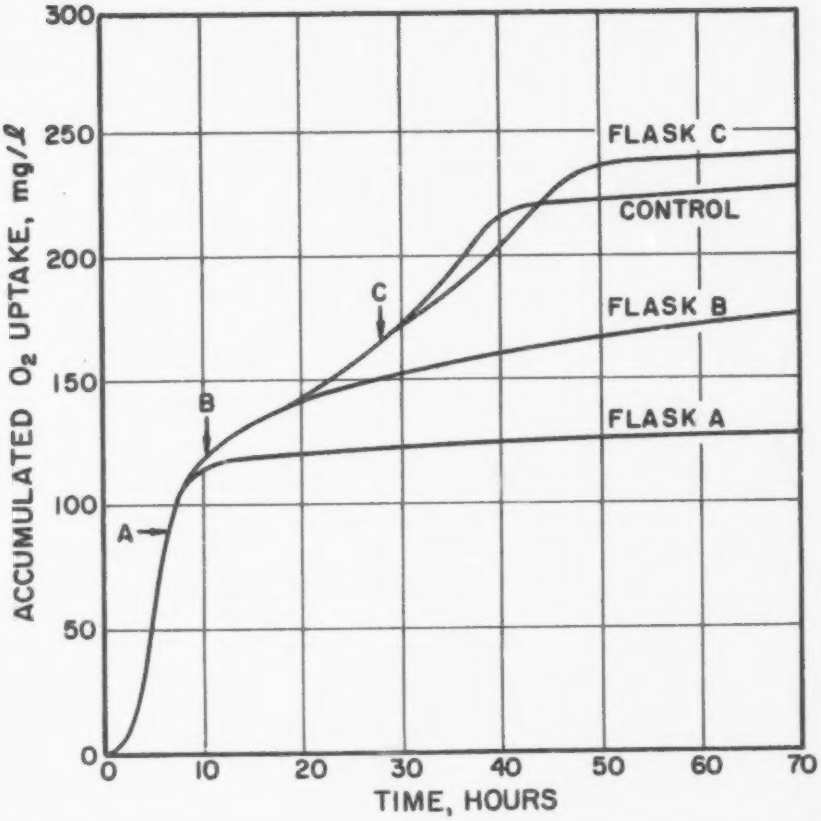

Fig. 6- EFFECT OF CHLORAMPHENICOL ON O_2 UPTAKE BY CULTURE NO. 13. (Arrows labeled A, B, and C indicate time of addition of chloramphenicol. All flasks contained 500 mg/ℓ glucose; the chloramphenicol concentration employed was 50μg/mℓ and temperature was maintained at 25°C. After nine hours glucose concentration was 9 mg/ℓ.

Endogenous Metabolism of Glucose-grown Cells

In Figure 3 it was seen that a secondary rise in oxygen uptake could occur in endogenous systems as well as in those to which substrate was added. It was felt that the study of endogenous metabolism of the cells could provide a useful comparison with the results of the previous experiments and might aid in determining the biochemical mechanism leading to production of the plateau. The course of oxygen uptake and changes in other parameters during the endogenous metabolism of culture No. 13 are shown in Figure 7. Diphasic oxygen uptake was observed in the absence of exogenous substrate. It is seen that there was a buildup of material responsive to the COD test in the medium until the end of the first phase of oxygen exertion; only a small percentage of this material was carbohydrate in nature, as determined by the anthrone reaction. The excreted material disappeared during the second stage of oxygen uptake. There was a decrease in cellular carbohydrate during the first phase of oxygen uptake, followed by a decrease in protein which coincided with the peak in filtrate COD and the pause in oxygen uptake. An overall loss of 250 mg/l dry weight of cells was recorded at the end of the experiment. Total cellular protein decreased 150 mg/l and cellular carbohydrate 120 mg/l.

DISCUSSION AND CONCLUSIONS

Culture No. 13, tentatively identified as Escherichia intermedia in the present study, was the only one of 57 pure cultures tested which showed a diphasic oxygen uptake with the two phases separated by a plateau. Thus it appears that there are not many commonly occurring bacteria which exhibit a plateau in oxygen uptake; however, the fact that a plateau in oxygen uptake for various pure cultures has been reported by Halvorson(8), Wilson and Harrison(6), Marion and Malaney(11), Gaudy, et al.(3), and in the present study indicates that such discontinuities in the kinetic course of oxygen uptake in the BOD bottle cannot be attributed solely to the heterogeneity of the population.

From the results presented in Figure 5 it seems apparent that the first stage of oxygen uptake was due to the incomplete oxidation of glucose to volatile acids. The second stage of oxygen uptake

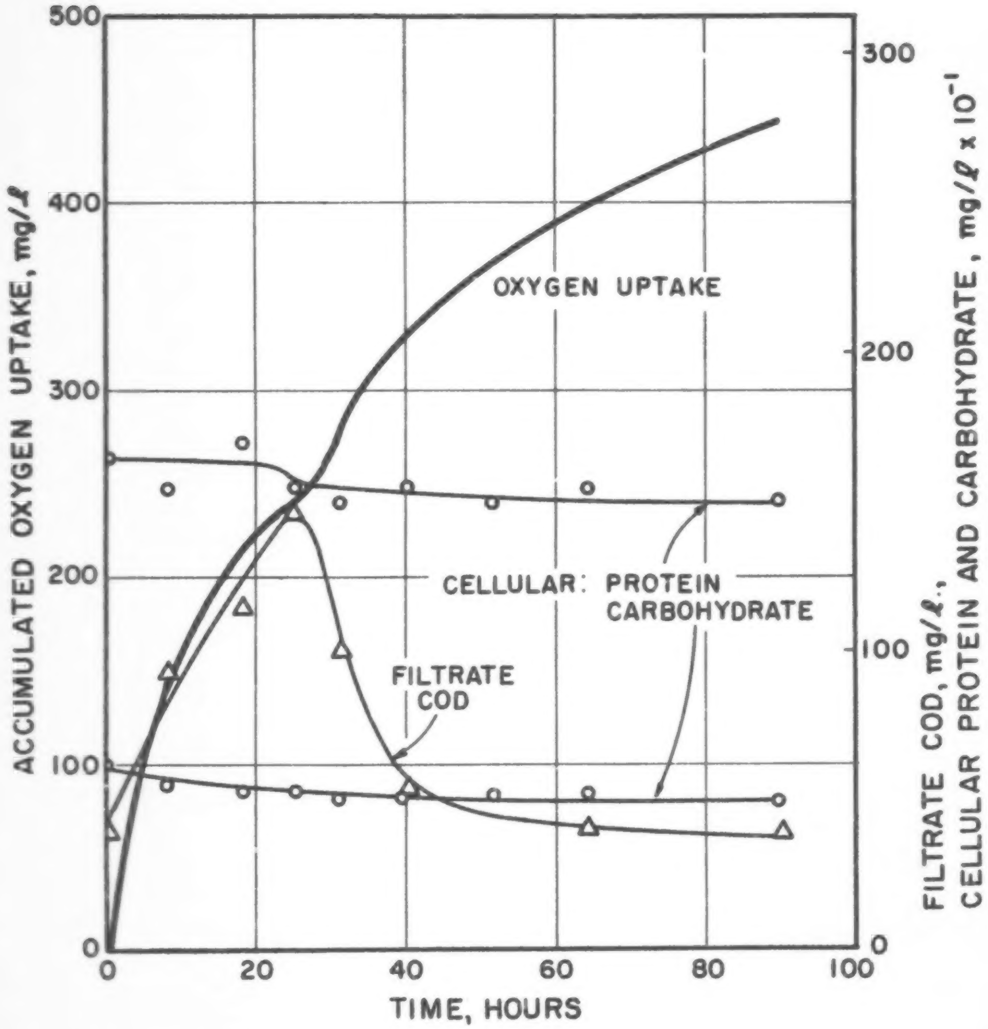

Fig. 7- ENDOGENOUS METABOLISM OF GLUCOSE GROWN CULTURE NO. 13. (Solids concentrations were 3285 mg/ℓ at 0 hours and 3035 mg/ℓ at 90.5 hours.)

corresponded to the disappearance of the volatile acids accumulated in the medium during glucose catabolism in the first phase. The utilization of volatile acids during the second stage of oxidation suggests that a new enzyme or enzyme system is induced during the transition period between the two phases. This explanation is substantiated by the studies reported in Figure 6. The blocking of enzyme synthesis, i.e., protein synthesis, by chloramphenicol in flask A before the point of total glucose removal completely prevented the occurrence of second-stage oxidation; however, the addition of the inhibitor 1.5 hours after total glucose removal in flask B reduced the respiration in the second stage compared to the control. The nearly linear (slightly decreasing) rate of oxygen uptake observed after treatment with chloramphenicol in flask B indicated that chloramphenicol did not inhibit the metabolism of volatile acids in cells that had already formed some enzyme(s). Linear or slightly decreasing rate kinetics would be expected in a system which contained a fixed or constant amount of enzyme. The modification in the secondary oxygen uptake in flask C, after tipping in chloramphenicol, may be due to stimulation of respiration caused by the inhibitor's interference with synthetic processes, thus resulting in higher ultimate oxidation. These findings point out that enzyme(s) synthesis was needed for the metabolism of secondary exogenous substrate, i.e., volatile acids. A quite similar situation has been reported with Bacillus cereus, which required the induction of a new enzyme system during the transition from vegetative growth to sporulation, the energy during the sporulation stage being derived from acetate(9).

It is suggested that the occurrence of the stepwise metabolism of glucose by culture No. 13 is due to one of the following:

(1) The acetate (volatile acids) utilizing system in these bacteria, as in many others, is an induced enzyme system (as indicated by the lag when grown in acetate, Figure 4), and formation of the required enzymes is repressed by glucose;

(2) A transport enzyme (a permease) is needed before the acetate can be brought into the cell for utilization and it is repressed in the presence of glucose.

The fact that the diphasic oxygen uptake curve was expressed only for culture No. 13 and in addition only with some substrates such as glucose, sorbitol and possibly ribose, points to the specialized nature of the biochemical mechanism responsible for this phenomenon. Logically, it may be reasoned that the absence of a plateau in the oxygen uptake curve with acetate and glutamate as substrates was due to the fact that the catabolism of these compounds did not involve the production of extracellular metabolic intermediates or end products to which acclimation was required. It can thus be surmised with considerable certainty that the plateau commonly observed during exertion of carbonaceous BOD, using a sewage seed, is not generally attributable to a particular metabolic characteristic of the bacteria. In general, the pattern of the utilization of oxygen by a single culture of bacteria is described by a phase of rapid oxygen uptake followed by a phase of slow endogenous respiration. Indeed, with most of the pure cultures studied, O_2 uptake broke very sharply. The percentage of the total oxygen used in the experiment after the break was very small, and it may be considered that there was really no second phase of oxygen uptake but simply a continuous plateau. Although it is the authors' conclusion that the occurrence of two stages of oxygen uptake separated by a plateau is not ascribable solely to bacterial metabolism, it is re-emphasized that various workers have observed plateaus for the carbonaceous BOD curve in purely bacterial systems and that it was essential to delineate both its cause and the generality of its occurrence.

These findings also indicate that caution must be exercised in interpreting the significance of the plateau and its predictive value in the BOD test. For heterogeneous populations the end of the first stage of oxygen uptake is usually found to occur approximately at the time of substrate removal. If the break in the uptake curve always occurred at a fixed percentage of the theoretical or stoichiometric oxygen demand for specific substrates or wastes, the plateau value could possibly be used as the means of estimating the theoretical oxygen demand of the waste. The use of the plateau to calculate this value has been suggested by Busch and Myrick(17); however, from the results of the present study it is seen that a plateau can be generated which definitely does not correspond to the point of total utilization of substrate. Furthermore, the authors have shown that plateaus can be generated by causes other than those shown in the present report(18). Therefore an analyst, running a time series of BOD's, cannot be sure if the plateau he observes corresponds to the time of removal of exogenous organic matter or is of the type shown to exist in these studies. Thus, his estimate could conceivably be in error by 100%.

It is of interest to compare the results of the detailed study of biochemical parameters associated with the plateau in O_2 uptake during endogenous respiration (Figure 7) with those of the fed system shown in Figure 5. In the endogenous system it was seen that during the first phase, while the cells were utilizing internal carbon stores, cellular materials were released. These materials were taken up during the second phase. In like manner, materials were released in the first phase and metabolized in the second phase in the fed system. Therefore, regardless of the source of the carbon (either exogenous or endogenous), the release of organic metabolites was associated with the first phase of oxidation and the second phase was associated with the utilization of these constituents, and probably also of endogenous materials which were not released. When an acclimation period is needed and is of sufficient length, it is manifested in the O_2 uptake curve and a plateau is generated. The plateau generated after the removal of exogenous carbon source (endogenous plateau) is naturally observed more easily at high cell concentrations wherein there are larger numbers of cells undergoing endogenous respiration.

The above analysis of the results shown in Figures 5 and 7 permits a more detailed analysis of the oxygen uptake curves shown in Figure 3 wherein various amounts of initial cell concentrations were employed. Here it was also seen that the endogenous flasks for the three highest cell concentrations exhibited plateaus in oxygen uptake and the magnitude of the second phase appeared to be proportional to the cell concentrations employed. In the fed systems it is seen that at the low initial inoculum (13 mg/l) there was a definite plateau which occurred slightly after 100 mg/l of O_2 had been utilized. Comparison of this curve with the one shown in Figure 5 (in which approximately the same initial cell concentration was used) leaves little doubt that the plateau was caused by the need for acclimation to carbonaceous material released during metabolism of the original exogenous carbon source. At the higher cell concentration this plateau was masked; however, it can be seen that for the 128, 256, and 640 mg/l cell levels there was a definite break in the O_2 uptake curve in the region of the observed plateau in the 13 mg/l system. This indicates that the higher numbers of cells allowed for the more rapid onset of uptake of the released materials, thus shortening the plateau to such an extent that it was manifested only as a break in O_2 uptake. However, it is seen that at the higher solids levels there was a second stage of uptake preceded by a plateau which, when viewed in light of the curves for the endogenous systems (Figures 3 and 7) is probably due, at least in part, to oxidation of materials released during endogenous metabolism. Thus, at some initial cell concentration between 13 and 128 mg/l it should be possible to register four phases of O_2 uptake and two plateaus. Indeed, a close scrutiny of the curve for the 128 mg/l cell inoculum indicates that there were four phases of O_2 uptake. The first ended after utilization of approximately 100 mg/l O_2; the second at slightly less than 200 mg/l; and the fourth began at slightly above 200 mg/l or approximately 55 hours on the time scale.

ACKNOWLEDGEMENT

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PAPER 11

SEQUENTIAL SUBSTRATE REMOVAL IN A DILUTE SYSTEM USING HETEROGENEOUS MICROBIAL POPULATIONS

By

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SEQUENTIAL SUBSTRATE REMOVAL IN A DILUTE SYSTEM USING HETEROGENEOUS MICROBIAL POPULATIONS

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ABSTRACT

The sequential metabolism of substrates by heterogeneous bacterial populations has been previously reported from this laboratory in studies using high substrate concentrations. This phenomenon has now been shown to occur at very dilute substrate concentrations, i.e., 5 mg/l glucose + 5 mg/l sorbitol, in studies conducted under the conditions of the standard biochemical oxygen demand (BOD) test. Sequential metabolism of these substrates resulted in a diphasic curve of accumulated oxygen uptake wherein the two phases were separated by a discernible plateau. These findings illustrate one possible explanation for the generation of discontinuity in the kinetic course of carbonaceous BOD exertion.

Work recently reported from this laboratory has shown that sequential substrate removal due to either repression of enzyme formation or suppression of enzyme function can occur in heterogeneous or natural microbial populations. (Gaudy, Gaudy and Komolrit, 1963) (Gaudy, Komolrit and Bhatla, 1963) (Gaudy, Komolrit and Gaudy, 1964). Since the previous work was accomplished in high energy systems, i.e., using fairly high concentrations of substrates and cells, it was primarily applicable to biological waste treatment systems.

It was of significant interest to determine whether repression would be detectably expressed in more dilute systems (low energy systems) such as usually exist in natural waters. In addition, one of the most important tests for measuring the oxygen required in the biological stabilization of organic wastes, the biochemical oxygen demand test (American Public Health Association, 1960) is necessarily carried out on dilute solutions. This test, in which a heterogeneous microbial seed is normally used, is universally employed for measuring the pollutional strength of waste waters, and is used for predicting the kinetics of oxygen depletion in polluted streams. However, there is much controversy concerning both the mechanism and the kinetics for the expression of carbonaceous oxygen demand, and it was felt that experiments on sequential substrate removal conducted under the conditions of the BOD test could give some insight into a possible cause of kinetic discontinuity in oxygen uptake as well as information on the occurrence of phasic substrate removal in dilute systems.

MATERIALS AND METHODS

In order to compare results with those previously reported in high energy systems, glucose and sorbitol were selected as carbon sources for the two-component synthetic waste in the present studies. From an initial inoculum of municipal sewage, a heterogeneous seeding material for use in the BOD bottles was developed on a laboratory shaker (100 osc/min, 25°C.) using the following minimal medium (concentrations in mg/liter): glucose, 3000; $NaNO_3$, 800; $MgSO_4 \cdot 7H_2O$, 200; $FeCl_3 \cdot 6H_2O$, 1.0; $MnSO_4 \cdot H_2O$, 20; $CaCl_2$, 15; 1.0M potassium phosphate buffer, pH 7.0, 30 ml; tap water, 100 ml; distilled water to 1 liter. The choice of nitrate over ammonia nitrogen was made in order to obviate the need to consider oxygen uptake due to nitrification by autotrophs (Gaffney and Heukelekian, 1958). After three serial transfers, the cells were harvested near the end of the log growth phase, and washed twice in 0.05 M potassium phosphate buffer, pH 7. The resultant cell suspension was then used to seed BOD dilution water prepared in accordance with Standard Methods (American Public Health Association, 1960). To one of two 20-liter carboys of dilution water, sufficient glucose and sorbitol were added to yield a final concentration of 5 mg/l of each carbon source. Both carboys were aerated for identical time periods; the seed suspension was added to both; dissolved oxygen was measured using the azide modification of the Winkler test (American Public Health Association, 1960). A series of BOD bottles for both the seed and seed plus sample carboys were filled by careful siphoning, and incubated at 20°C. At various times bottles for both series were removed and analyzed for dissolved oxygen concentration. The sample systems were also analyzed for substrate removal. The anthrone test was used for glucose and the periodate test for sorbitol (Gaudy, Komolrit and Gaudy, 1964). In order to determine the amount of substrate in these dilute solutions, the contents of a BOD bottle (approximately 300 ml) were passed through a membrane filter (Millipore Filter Corp., Bedford, Mass.; HA, 0.45μ) and the filtrate was concentrated under reduced pressure in a flash evaporator at 50°C. (Buchler Instruments, Fort Lee, N. J.).

RESULTS AND DISCUSSION

The results of a typical experiment are shown in Figure 1. It is seen that glucose and sorbitol were removed sequentially and that the sequential metabolism of these carbon sources was clearly reflected in the BOD exertion curve; i.e., diphasic oxygen uptake was observed. A similar experiment using cells acclimated to sorbitol also indicated sequential substrate removal in which glucose was removed first. When the glucose was nearly exhausted, sorbitol removal began rapidly at first, then proceeded at a slower rate. However, in this case sequential substrate removal did not result in a diphasic oxygen uptake curve. These results are consistent

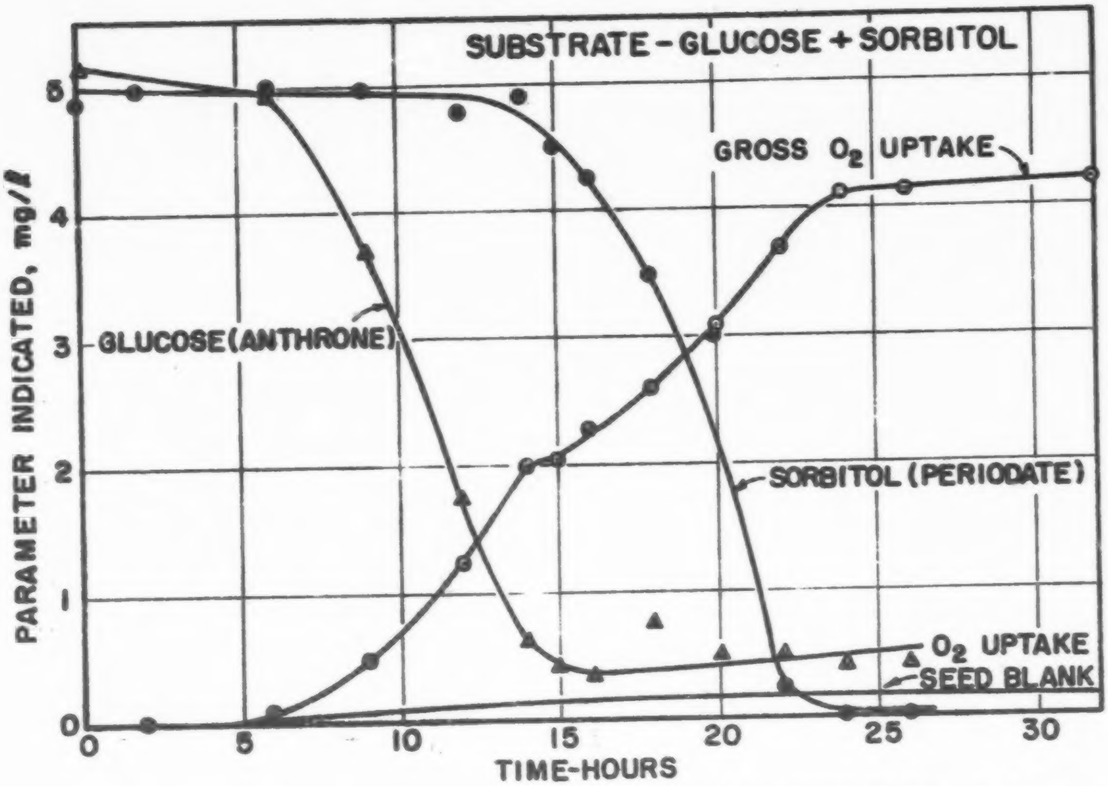

FIG. 1 - OXYGEN UPTAKE AND SUBSTRATE REMOVAL IN THE BOD BOTTLE, USING GLUCOSE-ACCLIMATED SEED.

with those observed in more exhaustive studies with high energy systems (Gaudy, Komolrit and Bhatla, 1963) and lead to the conclusions that there are no apparent differences in mechanism between high and low energy systems and that the repression mechanism requires only small concentrations of glucose (or other repressive compounds) in order to function in heterogeneous populations.

The demonstration of diphasic oxygen uptake due to sequential substrate removal has significant bearing on the BOD test itself. Many early investigators stated that, under conditions of the test, BOD exertion was described by decreasing rate, first order reaction kinetics. However, more recent investigations (Busch, 1958; Wilson and Harrison, 1960; McWhorter and Heukelekian, 1962; Gaudy, Bhatla and Abu-Niaaj, 1963) have shown that BOD exertion can proceed in two distinct phases of oxygen uptake separated by a pause or plateau similar to that in the accumulated O_2 uptake curve shown in Figure 1. This plateau or pause in oxygen uptake has been shown to occur in single carbon source systems as well as for systems containing many carbon sources. Some experiments have shown that the plateau in O_2 uptake occurs at the time of exhaustion of the exogenous carbon source (McWhorter and Heukelekian, 1962) (Bhatla and Gaudy, 1964). Busch (1958) proposed that this correlation could be successfully employed for devising a shortened BOD test procedure since the plateau usually occurs within the first day of incubation; that is, instead of the usual 5-day incubation period the test could be terminated when the plateau was reached. He also felt that the oxygen used up to the time of occurrence of the plateau was a fixed percentage of the total oxygen uptake theoretically possible for the particular substrate in question. Although the valid use of such a correlation is subject to doubt because of the difficulties of determining the "theoretical" oxygen demand for natural waste water, which represents a heterogeneous mixture of organic carbon sources, it could possibly be employed as an approximate means of estimating the total carbonaceous oxygen demand for certain specific waste waters. However, in systems which exhibit sequential substrate removal (see Figure 1) termination of the test at the apparent plateau period (approximately 15 hrs) would lead to erroneous results, since only half of the exogenous substrate had been removed at this time.

SUMMARY AND CONCLUSIONS

This study has shown that sequential substrate removal, previously reported for high energy systems, can also take place in very dilute systems. The generality of the phenomenon is again shown by the use of heterogeneous microbial populations; that is, the repressive mechanism causing sequential removal of the carbon sources is not a unique characteristic of relatively few species of bacteria. Also, from these results it would appear that a repressive compound need only be present in low concentration (0.03 mM) for its effect to be manifested in either sequential substrate removal or oxygen uptake. While these findings have a considerable basic significance, they have perhaps more important ramifications when applied to analyses of the BOD test. It is apparent that this test, which is one of the most widely employed microbiological analyses in water and waste water laboratories, does not always follow first order decreasing rate kinetics. Furthermore, the plateau value of O_2 utilization does not always correspond to the exhaustion of exogenous substrate.

ACKNOWLEDGEMENT

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PAPER 12

THE ROLE OF PROTOZOA IN THE DIPHASIC EXERTION OF BOD

By

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THE ROLE OF PROTOZOA IN THE DIPHASIC EXERTION OF BOD

By

M. N. Bhatla[1] and A. F. Gaudy, Jr.[2], M. ASCE

INTRODUCTION

The assessment of pollution is a very vital part of sanitary engineering practice, and the role played by the BOD test in this area is unique. The BOD test is widely used in the design and the operational control of treatment facilities. It is an important parameter in pollution surveys and in oxygen sag curve analysis. However, in contradiction to its wide application, the BOD test has been widely criticized because of lack of adequate understanding of the BOD reaction, and description of the kinetics of oxidation of carbonaceous material has been the subject of continuing controversy and research.

In recent years close examination of the course of BOD exertion has shown that oxygen uptake normally takes place in two distinct phases and that often a pause, commonly known as a plateau, exists between these two phases. However, the biochemical and biological causation for such kinetics has not been adequately explained.

The purpose of the present investigations was to delineate, insofar as is possible in heterogeneous populations, the cause(s) of diphasic oxygen uptake and, more specifically, to investigate

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This research was conducted in the bioengineering laboratories of Oklahoma State University, and represents a portion of the work submitted by M. N. Bhatla in partial fulfillment of requirements for the Ph.D. degree.

the mechanism(s) for the existence of the plateau or pause in the oxygen uptake curve between the two phases. It was further intended that more theoretically sound unifying or generalized concepts concerning BOD exertion would be sought through these studies. The major channel of effort involved the investigation of the four theories for the existence of the plateau which have been proposed in earlier reports from this laboratory[1]. The investigations were conducted in both high (Warburg studies) and low (the BOD bottle) energy systems.

Literature Survey

Busch[2] in 1958, working with pure soluble organic substrates, was the first to emphasize the occurrence of a plateau between two distinct phases of oxygen uptake. He ascribed the first phase of oxygen uptake to assimilation of the soluble nutrients, and the second phase to the summation of endogenous respiration of the bacteria and the respiration of predators feeding on the bacteria. Busch suggested that a higher initial number of protozoa in the seed would "blur" the separation of the two phases of oxygen uptake. He obtained slightly smaller BOD values when the seed was treated to reduce the protozoan population. The major purpose of Busch's work was the development of a short-term BOD test, and he proposed that the plateau value be used since he considered this to be more reproducible than longer-term oxygen utilization values. Although methods designed to reduce the protozoan population were employed, the only data obtained were oxygen uptake values, and there was no direct evidence for time of substrate disappearance or for kinetics of population changes.

In an attempt to test Busch's explanation pertaining to the contribution of protozoa to the second phase of oxygen uptake, Wilson and Harrison[3] demonstrated the existence of the plateau in Warburg studies using a pure culture inoculum and phthalic acid as substrate. They found that the magnitude of the second phase of oxygen uptake was greater when a higher initial inoculum of cells was employed. These workers felt that Busch's results could be explained by a reduction in bacterial numbers during the pretreatment of the inoculum to remove protozoa. They felt that the plateau might represent the delayed onset of endogenous respiration, but failed to state any reason why endogenous respiration should be so delayed.

In studies employing glucose as substrate, McWhorter and Heukelekian[4] observed the disappearance of the plateau at substrate concentrations of 1000 mg/l and above. They employed 0.5 per cent settled sewage as seeding material for all systems studied. In studies designed to assess the effect of protozoa, these investigators did not find any difference in the magnitude of oxygen uptake for sonicated and untreated seed systems until 45 per cent of the theoretical oxygen demand had been exerted. Since this was more than the plateau value for glucose (41 per cent) suggested by Busch, it was surmised that the disappearance of the plateau at high concentrations of glucose was not due solely to changes in the predator-bacteria ratio in the inoculum. However, no explanation was tendered for the disappearance of the plateau at high substrate concentrations.

One of the most widely quoted studies on the role of protozoa in the exertion of BOD is that of Butterfield, Purdy and

Theriault[5]. These workers noticed no difference in the shape of the BOD curve using either pure or mixed cultures of bacteria, except for a somewhat higher oxygen utilization in the latter case. In both cases a rapid phase of oxygen uptake which corresponded to bacterial multiplication was followed by a very slow phase. However, on the addition of the protozoan Colipidium to a pure culture of B. aerogenes, oxygen uptake continued much beyond the peak in the bacterial count and resulted in almost twice as much oxygen utilization as in the pure bacterial control. The bacterial population in the system to which the protozoa were added did not quite reach the count attained in the bacterial control, but it showed a rapid decline in bacterial population after one day. Following this there was an increase in the protozoan count which attained a peak after four days. The bacteria count in the control did not decline significantly after reaching a ceiling value. A plateau was not observed in these studies. Based upon these studies, Butterfield et al. advanced a theory stating that "the chief function of certain plankton in the biochemical oxidation process is to keep the bacterial population reduced below a saturation point and thus to provide conditions suitable for continuous bacterial multiplication, this, in turn, resulting in a more complete oxidation."

Zehnpfennig and Nichols[6] observed a decrease of nearly 30 per cent in a 5-day BOD when the protozoa were removed from sewage seed by straining.

Recently Javornicky and Prokesova[7] have studied the influence of protozoa on the course of BOD exertion. These investigators observed a considerable reduction in bacterial population during the development of protozoa in the BOD bottle. During this period there was an increase in oxygen uptake. In studies using "partially sterilized water" in which the predators had been selectively killed, they noticed higher bacterial counts and lower oxygen uptake values. They felt that their results were explained by the hypothesis of Butterfield et al. These workers also made studies in which a pure culture inoculum of the protozoan Tetrahymena pyriformis was placed into a mixed bacterial system. This species of protozoa did not flourish and did not affect oxygen uptake. In other experiments flagellated protozoa ranging up to 10 µ in size caused only a slight decrease in bacterial number and a very small increase in oxygen consumption. From these studies it was felt that both the quality and the quantity of protozoa in the inoculum are important in the determination of the BOD. The oxygen utilization data presented showed considerable fluctuation and no rigid treatment of BOD kinetics could be made.

Gaudy, Bhatla and Abu-Niaaj[1], and Follett and Gaudy[8] have observed a correlation between the different phases of BOD exertion and changes in the viable bacteria population. After many studies it was concluded that, in general, the first phase of rapid oxygen uptake was accompanied by an increase in bacterial numbers. The plateau corresponded to the range of the maximal, or steady, bacterial population, and the secondary rise in oxygen uptake was marked by a decrease in viable bacteria. It was found that the plateau occurred with a wide variety of substrates and seeds, including a pure culture of Escherichia coli.

From the results of all the studies thus far cited it can be seen that there is considerable disagreement concerning the plateau in BOD exertion. It is apparent that involvement of protozoa is not required for the occurrence of a plateau in oxygen uptake, since they have been observed using pure cultures. Since the BOD test is of primary importance in the pollution control field, it was felt that work should be undertaken to obtain definitive data concerning the mechanisms of BOD exertion which are the underlying cause for variations in BOD kinetics. Based upon studies described above[1, 8], four related theories for the causation of the plateau were presented. Since the present report bears upon the validity of these theories, they are briefly recounted below.

THEORETICAL CONCEPTS

The present study represents the concluding phases of investigations initiated to delineate the causes for the occurrence of the plateau during the exertion of BOD. The investigation was systematically undertaken by testing the applicability of the four theories for the causation of the plateau previously proposed by the authors. These theories are as follows:

1) Changes in the predominance of species in a heterogeneous population may occur, and the time required for the shift in predominance may be expressed as a plateau in oxygen uptake. After the removal of the original exogenous substrates (which in general takes place in the first phase of oxygen uptake), a major change in predominance may take place. The new or secondary predominating cells may be either bacteria or predators such as protozoa. In the former case, the food for the secondary predominant(s) is provided by lysis products of the primary predominant(s), while in the latter case the intact cells are metabolized by the predators.

2) There may be no change in predominating species, but a

portion of the cells which have grown up in the first phase of oxygen uptake may die and release products to the medium which, after an acclimation period, may be metabolized by the remaining viable population. This acclimation period results in a lag in oxygen uptake before the second phase is initiated, and appears as a pause or plateau in the total oxygen uptake curve.

3) After removal of the exogenous substrate and attainment of maximum viable population in the first phase of oxygen uptake, the environmental conditions may prevent further replication but the cells may continue to respire. An acclimation period may be needed for the purpose of synthesizing new enzyme systems required for endogenous metabolism of various cell materials, such as certain carbohydrates, lipids and protein. This acclimation period is reflected as a plateau in the oxygen uptake curve.

4) While the original exogenous compound is being rapidly metabolized in the first phase of oxygen uptake, new compounds consisting of cellular intermediates or metabolic by-products may be released into the medium. An acclimation period may be required before these compounds can be metabolized. This induction or lag period may cause a plateau in oxygen uptake.

In addition to the four causes listed above, Gaudy, Komolrit and Bhatla[9] have shown that a diphasic oxygen uptake curve can be produced in high energy systems due to the sequential utilization of exogenous substrates in multicomponent media. More recently Bhatla[10] has shown this effect in dilute systems (the BOD bottle). However, this mechanism of causation does not apply in systems employing a sole carbon source, and since most of the studies in which the existence of the plateau is reported employ such systems, sequential substrate removal was not considered to be a general mechanism of causation for the plateau during the BOD test.

Concerning the four theories given above, the authors believed that all or any one of them could contribute to the production of a plateau in some systems. Theory 1 applies only to heterogeneous populations; however, it is such populations which are of primary importance in the water pollution control field. In seeking general causation for the plateau it was therefore desirable to direct most of the research effort toward understanding the phenomenon in such natural populations.

In a recent report from this laboratory, it was concluded that Theories 2 and 4 may be eliminated from consideration as general causes for the plateau in heterogeneous populations[11]. This work was accomplished in high energy systems (Warburg apparatus) using a glucose synthetic waste water. The work herein reported was conducted in continuation of the work described above, and the research emphasis was placed upon the study of those mechanisms within the scope of Theories 1 and 3.

EXPERIMENTAL APPROACH

A. Examination of Theory 3

Theory 3 recognizes the requirement for an acclimation period during which new enzyme(s) are synthesized. Therefore, if protein synthesis could be artificially inhibited before or during the second phase of oxygen uptake, a considerable insight into the applicability of Theory 3 could be gained. For the present study chloramphenicol was an ideal inhibitor of protein synthesis because it has been shown to affect neither the fermentation nor the respiration of glucose, even at very high concentrations[12, 13]. Chloramphenicol is essentially a bacteriostatic agent, which has been shown to be effective in inhibiting protein synthesis in a large variety of bacteria at concentrations between 1 and 10 mg/l[14,15]. It was also ideally suited to these studies, since most protozoa are very resistant to chloramphenicol[14]. Tetrahymena pyriformis, which is inhibited at a concentration of 40 mg/l, is an exception[16]. It has been suggested that the inhibitory mechanism of chloramphenicol involves interference with processes of transfer of amino acid from soluble RNA to protein during protein synthesis[17]. Preliminary experiments indicated that chloramphenicol was effective in inhibiting protein synthesis in heterogeneous bacterial populations, and that it did not interfere with the growth of the protozoa[10].

B. Examination of Theory 1

According to Theory 1 the second phase of oxygen uptake may be caused by a secondary predominating species (bacterial or protozoan) subsisting on products made during the first stage of oxygen uptake. While one way of studying this mechanism would have been to use mixtures of known species, it is the natural heterogeneous population which is of primary interest in the water pollution control area, and a variety of experiments was designed for the direct study of Theory 1 using such populations. Methods for selectively inactivating the protozoa were perfected, as well as a technique for counting protozoa. The effect of the protozoa-bacteria relationship in the inoculum on oxygen uptake was studied in both low and high energy systems. The effects of adding protozoa to purely bacterial systems were also examined with respect to oxygen uptake and other biological parameters.

EXPERIMENTAL

The composition of the synthetic waste water used in these studies is given in Table I.

TABLE I

COMPOSITION OF SYNTHETIC WASTE WATER

Constituents	Quantities per Liter
Glucose or other Carbon Source	500 - 3000 mg*
1.0 M Potassium Phosphate Buffer pH 7.0	30 ml
$NaNO_3$	800 mg
$MgSO_4 \cdot 7H_2O$	200 mg
$FeCl_3 \cdot 6H_2O$	1 mg
$MnSO_4 \cdot H_2O$	20 mg
$CaCl_2$	15 mg
Tap Water	100 ml
Distilled Water	To make one liter

*3000 mg/l concentration was used for obtaining acclimated seed.

For most of the Warburg studies a glucose concentration of 500 mg/l and a small initial seed inoculum was used. For all experiments 125-ml reaction flasks were employed; temperature was maintained at 25°C. and the shaker rate at 100 oscillations per minute. For experiments conducted in dilute systems (the BOD bottle) the standard dilution technique was employed[18], and dissolved oxygen was determined by the Alsterberg modification of the Winkler method. Sterile deionized water was employed in making up the dilution water. For study of substrate removal in the BOD bottle, samples were concentrated 40-fold in a flash evaporation apparatus at 50°C.

Population Estimate

The viable bacteria count was estimated using the surface plating technique[19] with nutrient agar as the growth medium. The protozoan population was estimated by direct observation at 125x magnification, using a Howard mold counting chamber (15 mm x 20 mm x 1/10 mm). Only motile protozoa were counted, since this was the only practical method for distinguishing viable cells. In all cases the majority of protozoa observed were motile species. During each counting operation 25 random fields (1.5 mm^2 each) out of a possible 200 fields were considered. All protozoa and bacteria counts were made in duplicate, and the results reported are the average of the duplicates.

Other Parameters

Glucose removal from the medium was followed by the anthrone determination[20], and the biological solids production was measured by the membrane filter technique[18]. Cellular protein was determined using a modification of the Folin method[10].

Seed Material

The types of seed inocula used were as follows:

a) Settled sewage seed

b) An acclimated seed, developed from an initial sewage seed; the culturing technique was the same as that previously employed[11].

c) Pasteurized seed. A protozoan-free seed, prepared by pasteurization of sewage seed at 50°C. for five minutes[21,6].

d) Diluted seed. This seed was prepared in order to obtain various protozoa-bacteria ratios in the initial inoculum. This was achieved by diluting the initial protozoa population in the seed.

The following formula was used for preparing the diluted seed:

$$S_x = 2 \text{ ml pasteurized } S + 2 \text{ ml } S \text{ diluted } \frac{x}{2} \text{ times with}$$
$$0.05M \text{ phosphate buffer, pH } 7$$

where S is sewage seed and x is a protozoa dilution factor with respect to the original seed (which in this case is sewage seed).

e) Protozoa-rich seed. In the preparation of seed containing a high protozoa count, 2 ml of settled sewage were placed in 60 ml of salts medium (Table I) containing 500 mg/l of glucose. This system was aerated on a laboratory shaker at 100 strokes per minute for 48 hours. The temperature was maintained at 25^{o}C. A seeding inoculum taken from this mixed liquor was designated as a protozoa-rich seed; preliminary experimentation indicated that this procedure provided seed with a fairly high number of protozoa.

EXPERIMENTAL RESULTS

A. Investigations Related Directly to Theory 3

Figure 1 shows oxygen uptake curves for a glucose synthetic waste inoculated with 0.5 per cent sewage seed. Chloramphenicol in concentrations of 10 and 50 mg/l was added to the mixed liquor well before the break in oxygen uptake, i.e., before the plateau (see arrows). The biological solids concentration of the mixed liquor and both the COD and carbohydrate contents of the filtrate were determined at the point of addition of chloramphenicol and are shown on the figure. A control system which received no chloramphenicol is also shown. It is seen that the addition of the protein synthesis inhibitor at either concentration employed did not significantly affect the development of the plateau or the second stage of oxygen uptake, even though a sufficient amount

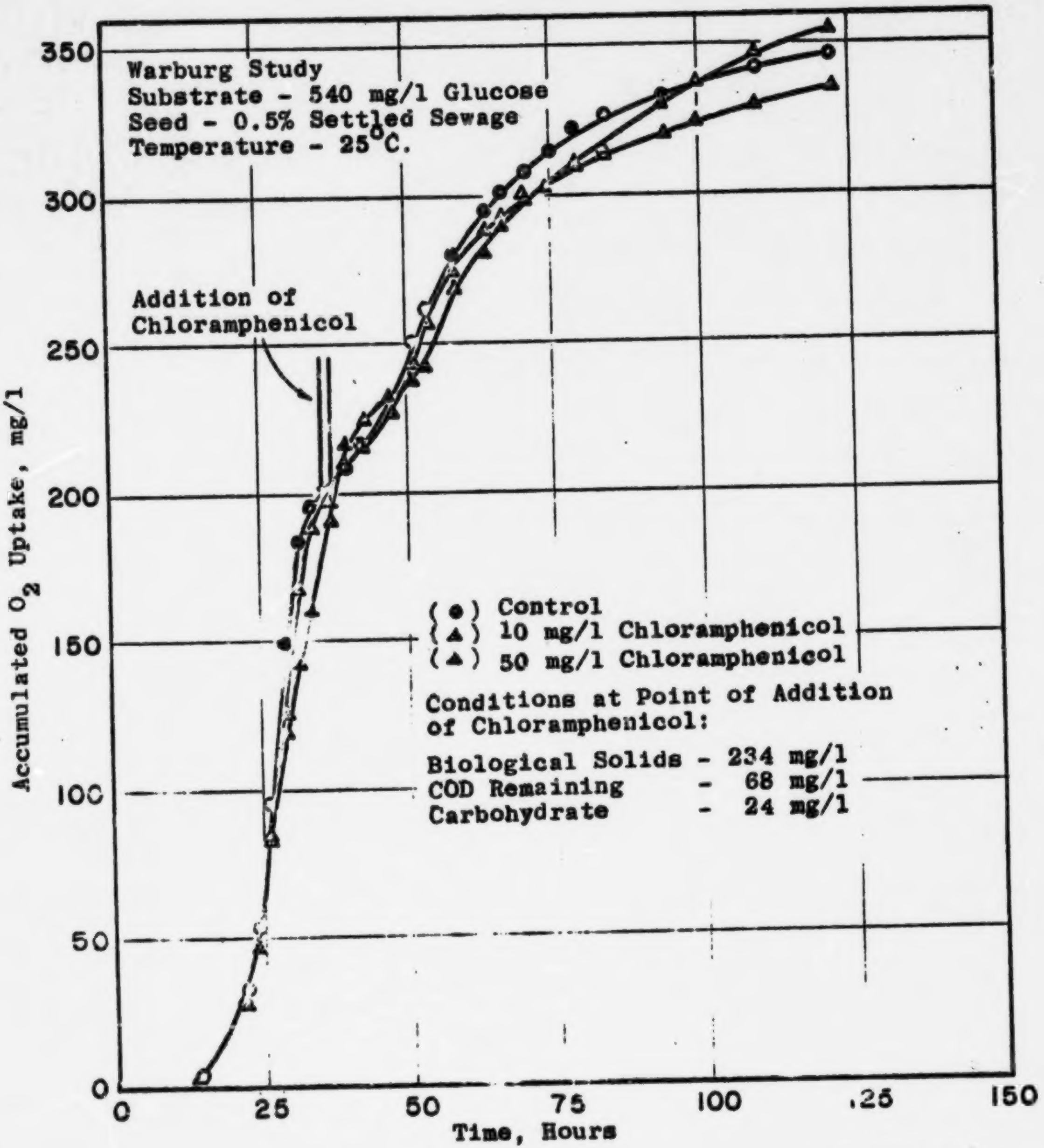


Fig. 1 - EFFECT OF CHLORAMPHENICOL ON O_2 UPTAKE USING SEWAGE SEED.

of chloramphenicol had been added to completely inhibit bacterial protein synthesis.

B. Investigations Related to Theory 1

Preliminary Warburg studies indicated that the second stage of oxygen uptake was practically eliminated when pasteurized seed was used. Also, it was noted that the filtration of a sewage seed through Whatman #1 paper modified the O_2 uptake curve in two ways:

1) A clear separation of the two stages of oxygen uptake by a plateau was always noted.

2) Filtration invariably reduced the magnitude of the second stage of oxygen uptake.

The results of experiments such as these indicated that the role of protozoa might not be simply to blur or mask the plateau, as suggested by Busch, but that the protozoa could actually be the major cause of the second stage of oxygen uptake. Definitive experiments were designed to investigate this possibility.

Filtration Studies

The most minute protozoa known belong to the family Monadidae and range in length from 2 to 10 μ. Thus microfiltration of the seed provided a suitable technique for excluding protozoa without causing a restrictive selection in the bacterial population such as might result from exposure to heat during a pasteurization procedure. Figure 2 shows the course of oxygen uptake using untreated sewage seed and sewage seed filtered through membrane filters of varying pore size. It is seen that the 1.2 μ and 3 μ filters caused the second stage of oxygen uptake to be eliminated, whereas a very long plateau was evidenced for the system filtered through

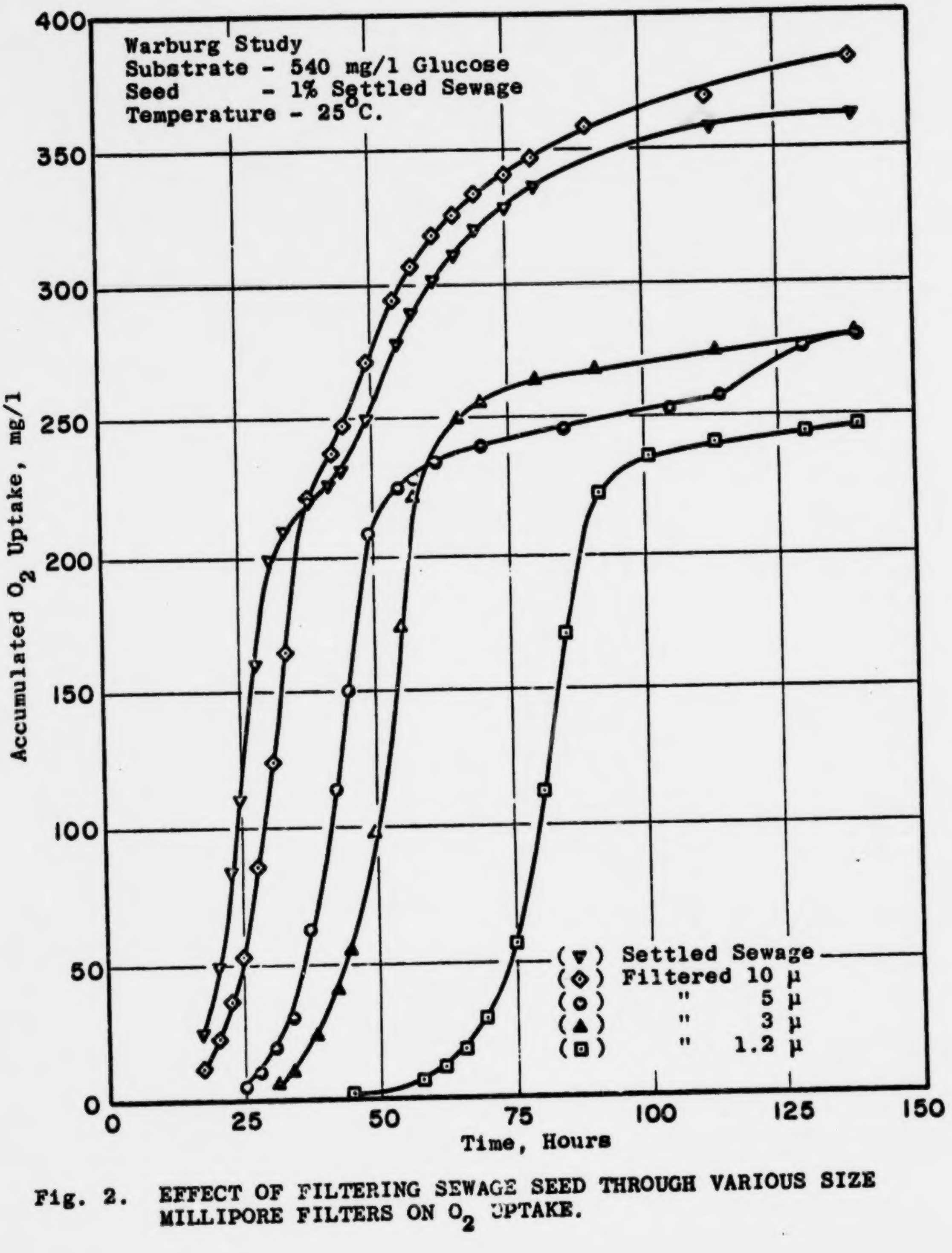


Fig. 2. EFFECT OF FILTERING SEWAGE SEED THROUGH VARIOUS SIZE MILLIPORE FILTERS ON O_2 UPTAKE.

the 5 μ filter. Straining the seed through the 10 μ filter produced a slight plateau but had little effect on ultimate oxygen consumption.

At the termination of the experiment, microscopic examination revealed that primarily flagellated and highly motile protozoa were present in both the sewage seed control and the 10 μ system. Many less protozoa were observed in the 5 μ system, and absolutely no protozoan activity was noted in either of the remaining flasks. It was also noted that the turbidity of the reaction liquor was much lower in the sewage seed control and the 10 μ system than in any of the others.

These results suggested that the second stage oxygen uptake was due almost entirely to the metabolic activity of the protozoa and that most of the protozoan population responsible for the second stage of oxygen uptake could be retained on the 5 μ filter.

Population Dynamics during Oxygen Uptake

Figures 3 and 4 show changes in numbers of bacteria and protozoa during oxygen uptake in the Warburg apparatus using 2 per cent sewage seed (Figure 3) and 2 per cent pasteurized seed (Figure 4). In Figure 3 it is seen that the break in the oxygen uptake corresponds to the region of maximum viable bacteria count. There was a slight plateau in oxygen uptake which corresponded to a stationary viable bacteria count, and the second stage of O_2 uptake was marked by a rapid increase in the protozoan population and a rapid decline in the viable bacteria count. The attainment of the maximum protozoan population lagged the peak in bacterial count by approximately 15 hours, and at the time of attainment of

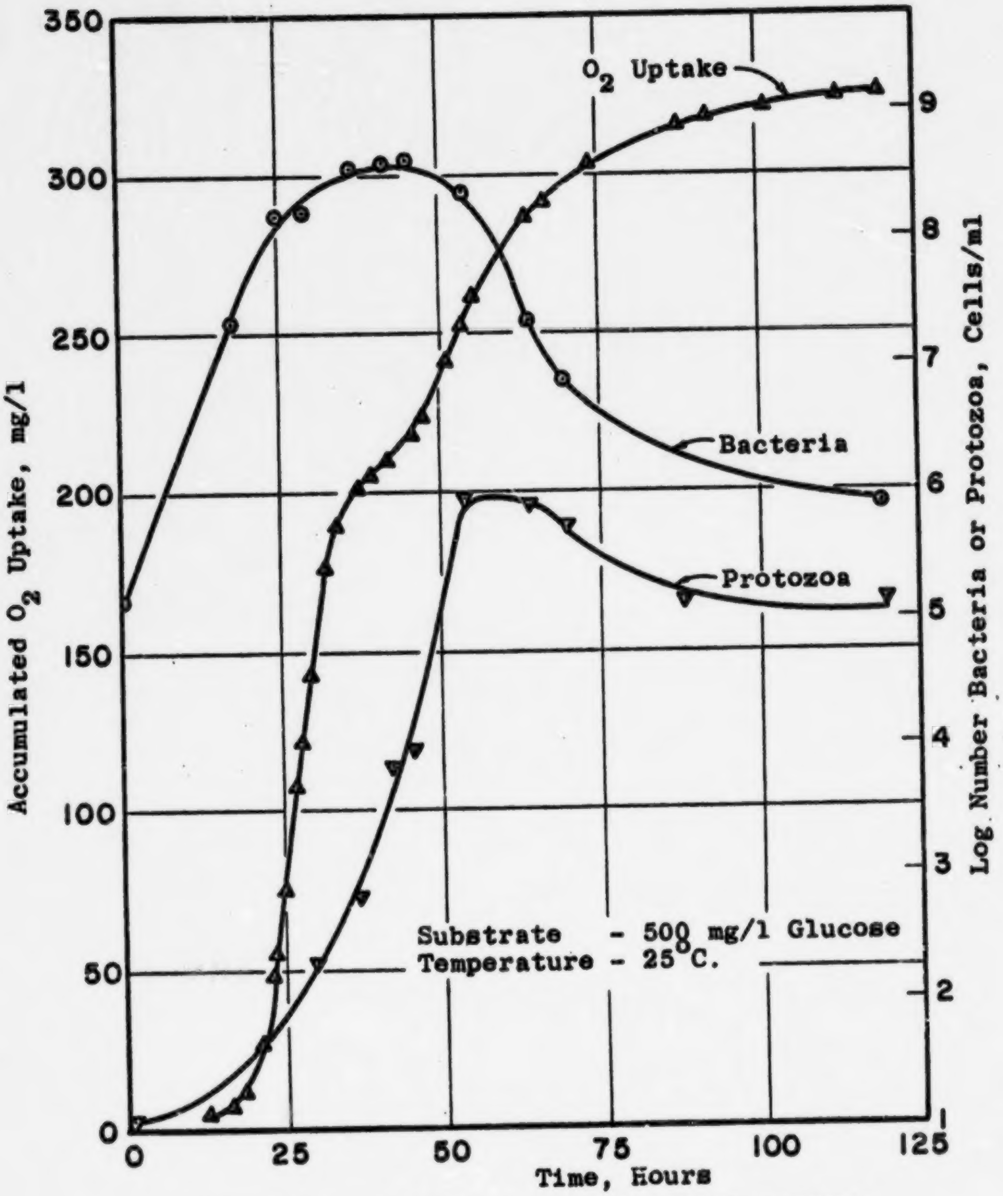


Fig. 3 - BACTERIA AND PROTOZOA COUNTS DURING WARBURG STUDY USING 2% SEWAGE SEED.

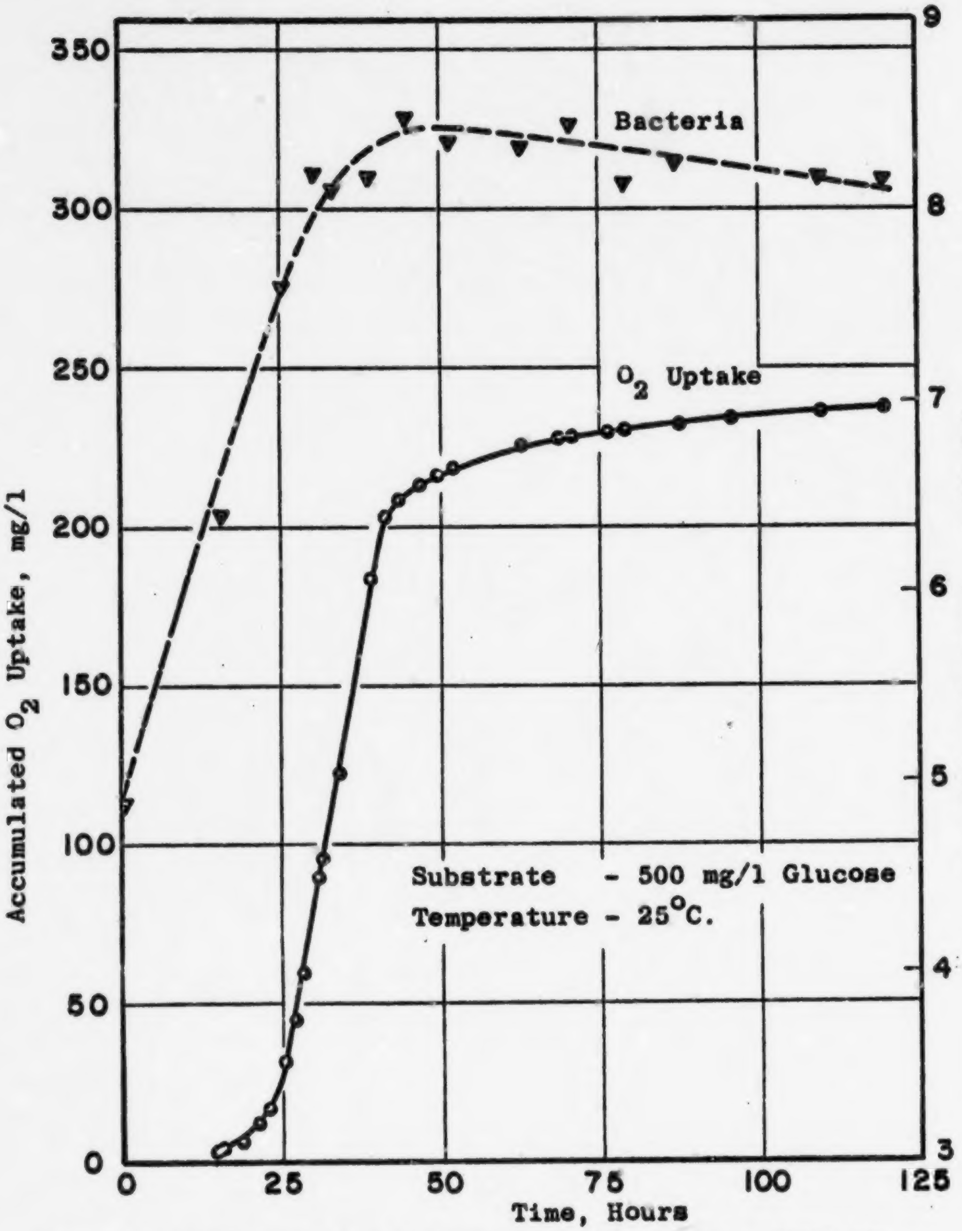


Fig. 4 - BACTERIA COUNT DURING WARBURG STUDY USING 2% PASTEURIZED SEWAGE SEED.

maximum protozoan density slightly less than half of the second stage oxygen uptake had been expressed. Microscopic examination showed that flagellated protozoa belonging to the genus Monas predominated. Enchelys and Colpidium were also present in much smaller numbers. In contrast to these results, the pasteurized seed system shown in Figure 4 did not exhibit a second phase of O_2 uptake, and the amount of oxygen used corresponded to that of the first phase shown in Figure 3. Again, the highest bacterial density was attained at the break in oxygen uptake; however, it remained at this high level for an extended period of time. Microscopic examination showed a complete lack of protozoan activity throughout the duration of the experiment. Also, the system was much more turbid than that of Figure 3.

Similar experiments at much lower substrate concentrations were conducted using the standard BOD dilution technique. A typical experiment is shown in Figure 5. It is seen that the same result was obtained in the BOD bottle experiments as in the higher substrate level studies conducted in the Warburg apparatus. It is important to note that the plateau shown for the sewage seed in Figure 5 was of longer duration than that shown for a similar seed in Figure 3, and that there was a greater time period between attainment of maximum bacterial density and maximum protozoan population. It is also apparent from Figure 5 that the first stage of oxygen uptake is attributable to bacterial growth whereas the second stage is attributable to the growth of the protozoa.

A typical BOD experiment using sewage seed in which substrate removal was also assessed is shown in Figure 6. It is seen that the break in oxygen uptake occurs concurrently with the time of

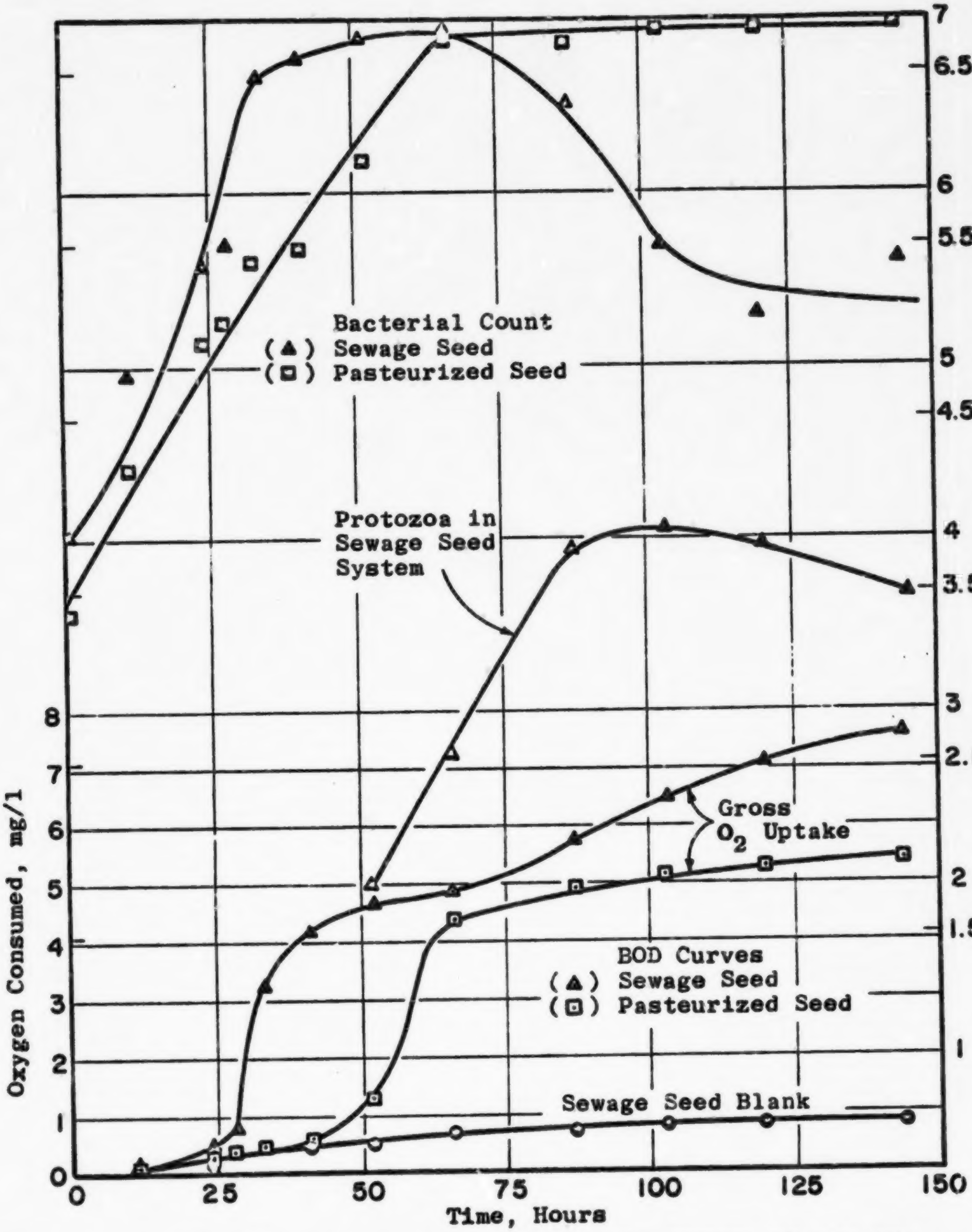


Fig. 5 - BOD EXERTION, BACTERIA AND PROTOZOA COUNTS IN THE BOD BOTTLE, USING 11 mg/l GLUCOSE.

maximum viable bacteria count and exhaustion of the exogenous substrate. It is important to note that the protozoa appeared quite late in the experiment, and showed a rather slow development toward maximum population. The rather long time period between attainment of maximum bacterial and protozoan populations is reflected in the development of a long plateau in the oxygen uptake curve.

Figure 7 shows the results of an attempt to compress the plateau during the BOD test by using a higher number of protozoa in the seed inoculum than was usually present in the sewage seed. The seed for this experiment was obtained in accordance with the procedure described for the "protozoa-rich seed" preparation. It is seen that a considerably shorter plateau was developed than that shown in Figure 6. Figure 7 also shows that pasteurization of the protozoa-rich seed eliminated the second stage of O_2 uptake but had little or no effect on the magnitude of first stage oxygen uptake.

From the results presented above it would appear that in heterogeneous populations the presence of protozoa is essential for the exertion of a second stage of oxygen utilization. The first stage is almost wholly attributable to the growth of the bacteria. However, the occurrence of a plateau between the two phases and its severity depend upon the relative displacement of the growth curves of the bacteria and the protozoa in the system, i.e., the length of the plateau is governed by the time lag between the peaks in bacterial and protozoan populations.

The Effect of the Protozoa-Bacteria Ratio of the Seed Inoculum on Oxygen Uptake

A seed dilution method was employed to prepare seeding material which contained successively smaller numbers of protozoa.

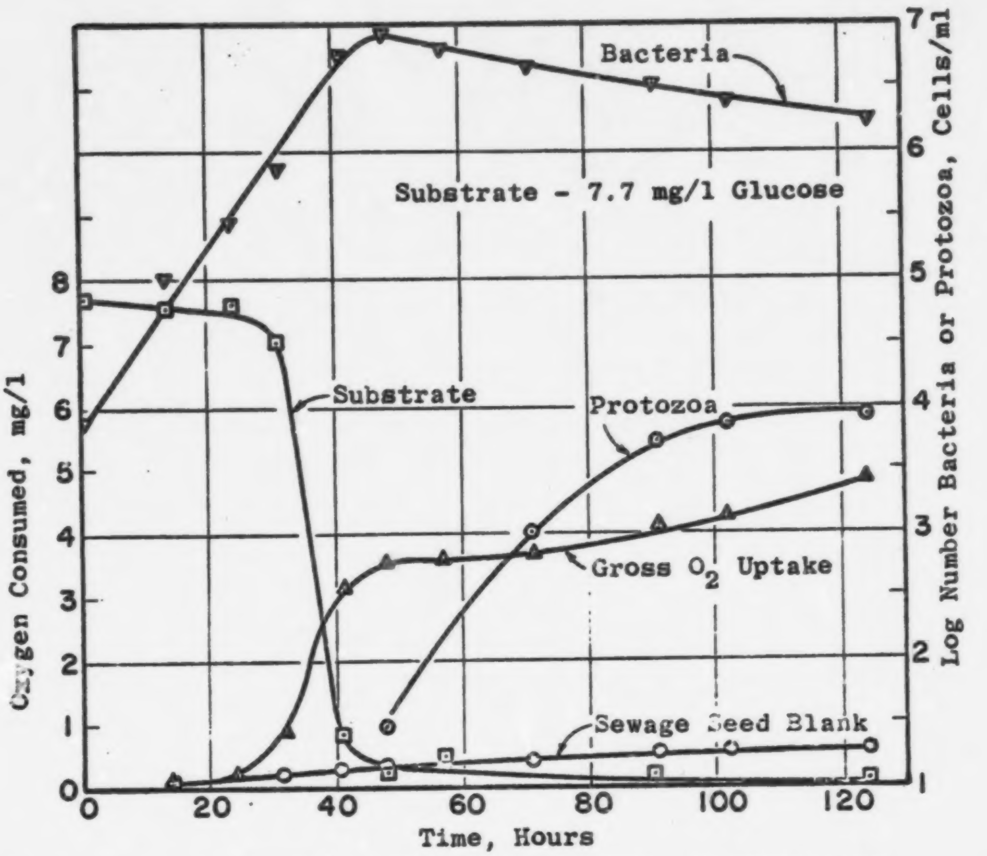


Fig. 6 - BOD EXERTION, SUBSTRATE REMOVAL, BACTERIA AND PROTOZOA COUNTS IN THE BOD BOTTLE USING SEWAGE SEED.

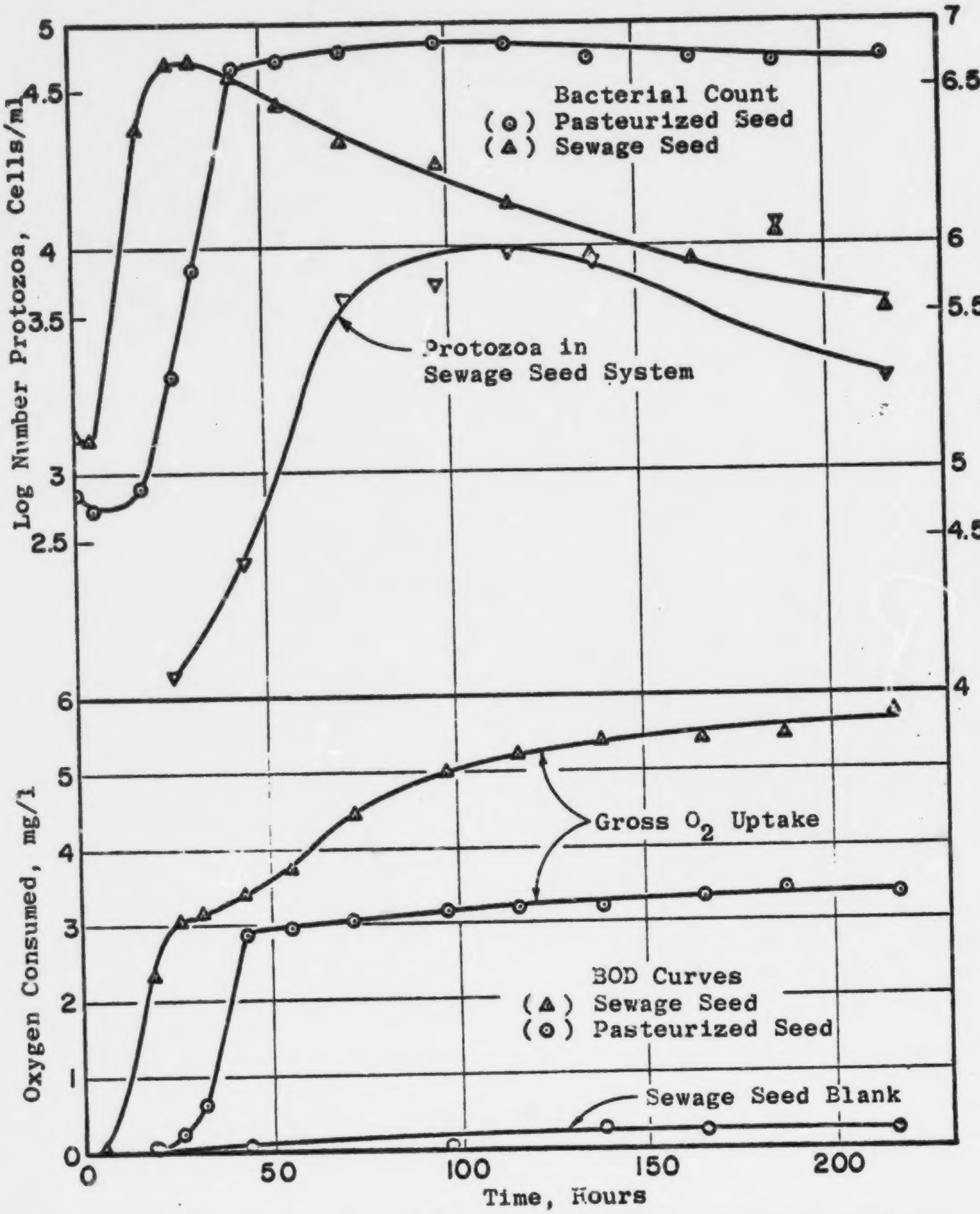


Fig. 7 - BOD EXERTION, BACTERIA AND PROTOZOA COUNTS IN THE BOD BOTTLE USING 8 mg/l GLUCOSE.

In order to maintain approximately the same bacterial numbers in the final seed material, a pasteurized seed was used as a base to which different dilutions of the protozoa-containing seed were added. The procedure for obtaining the seed material was described previously under the heading "Diluted Seed." The letter "S" is used for the seed preparation derived from the sewage seed, and the subscript number indicates the dilution factor with respect to protozoa as compared to the parent sewage seed; e.g., S_5 indicates a seed containing five times less protozoa than the sewage seed. The effects on oxygen uptake using various protozoa-bacteria ratios in high energy (high substrate) systems are shown in Figure 8. While the S_5 and S_{10} seed caused increasing extensions of the plateau, the S_{100} and S_{200} protozoan dilutions caused complete elimination of the second stage of oxygen utilization during the 5-day experimental period. The results obtained with the two higher dilutions were comparable to those using pasteurized seed.

A similar elongation of the plateau was evidenced using S_{10} seed in experiments conducted at low substrate levels (in the BOD bottle). In Figure 9 results using S_{10} seed are compared with those obtained using nondiluted sewage seed and seed for which the protozoa had been totally "diluted out" (pasteurized seed). The oxygen utilization during the second stage by the system containing a lower number of initial protozoa was less than in the sewage seed system. Also, the bacterial count decreased less rapidly and to a lesser amount for the diluted seed (S_{10}) as compared to the sewage seed. The system for which pasteurized seed was employed showed the least second stage oxygen uptake, and the viable bacteria count did not decrease after reaching a maximum number.

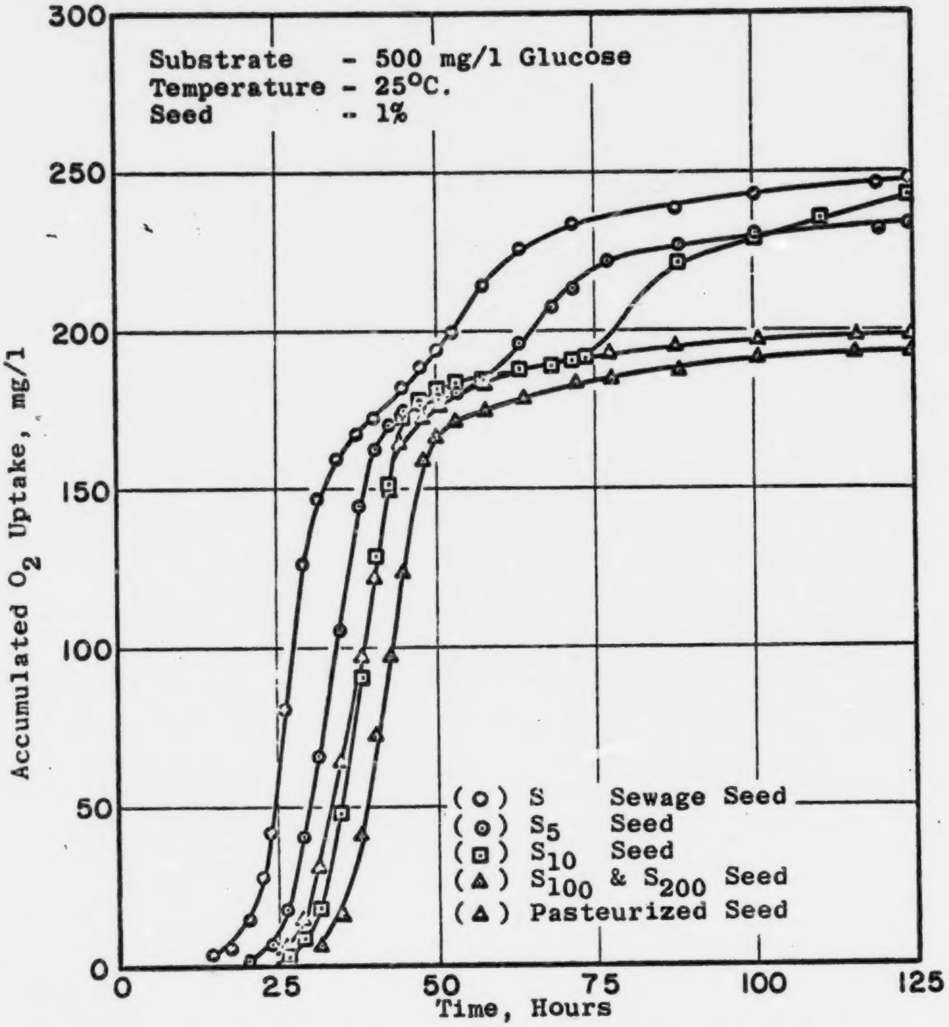


Fig. 8 - WARBURG STUDIES USING DIFFERENT PROTOZOA-BACTERIA RATIOS IN SEED PREPARATIONS.

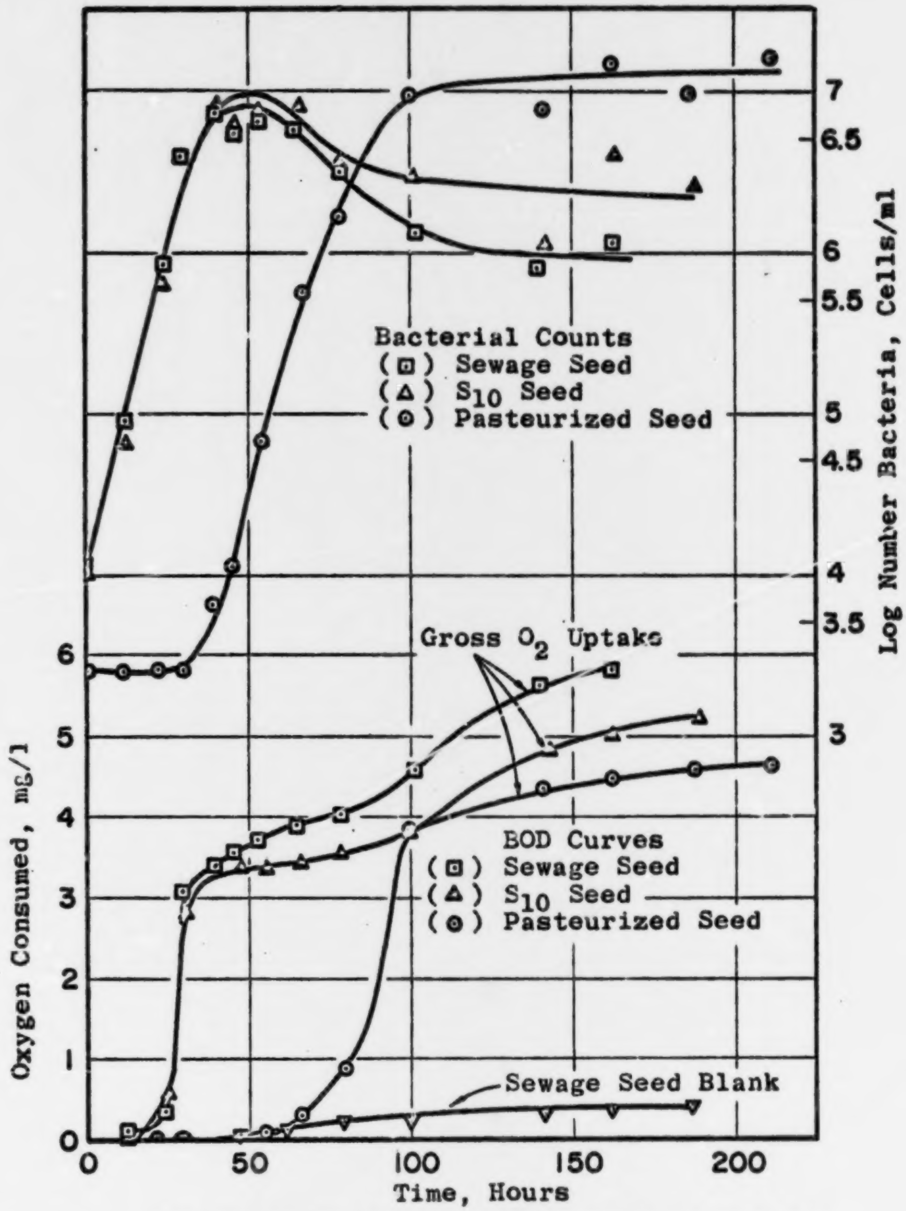
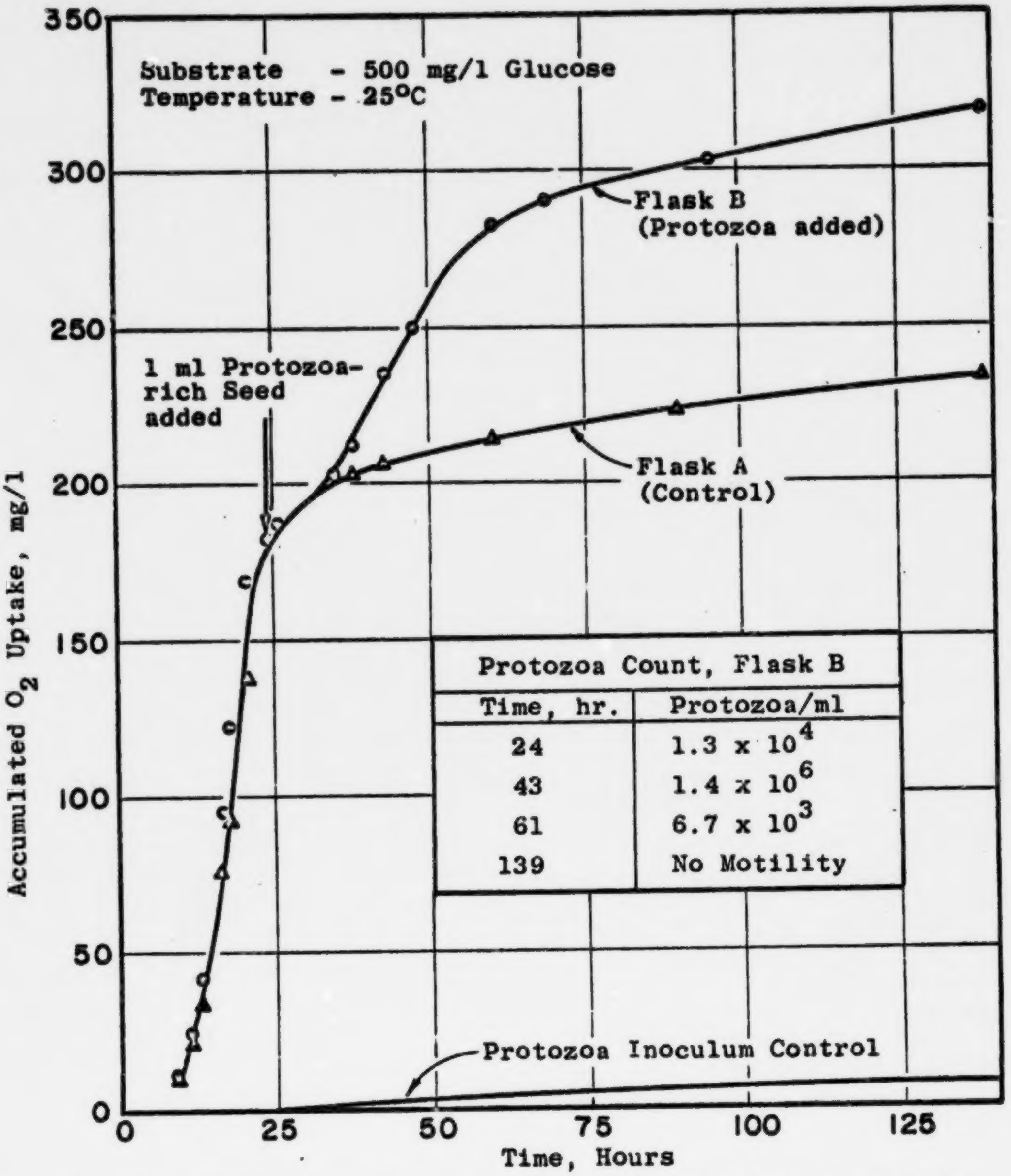


Fig. 9 - BOD EXERTION AND BACTERIA COUNT IN THE. BOD BOTTLE, USING DIFFERENT PROTOZOA-BACTERIA RATIOS IN SEED PREPARATION. GLUCOSE CONCENTRATION, 9 mg/l.

Effect of Inoculations of Protozoa-Rich Seed on Oxygen Uptake

The effect of predation upon the production of the plateau and the amount of oxygen utilization in the second stage by the predators was studied by adding a protozoa-rich inoculum to a heterogeneous bacterial culture during the course of oxygen uptake. A series of Warburg flasks containing 500 mg/l glucose synthetic waste (Table I) was inoculated with a pasteurized acclimated seed and the course of oxygen uptake was followed. At the end of 24 hours (see arrow, Figure 10) when rapid oxygen uptake had ceased, a number of flasks were inoculated with 1 ml of the "protozoa-rich" seed. Flask A was followed as a control. It is seen in Figure 10 that a secondary oxygen demand was exerted in systems which received the protozoa inoculation. Flask B consumed 97 mg/l of oxygen in excess of the control flask A, indicating that approximately 30 per cent of the total oxygen consumption in flask B was due to the addition of the protozoa. Flasks treated similarly to flask B were removed to obtain the protozoa count. It is seen that the protozoan population in flask B increased from 1.3×10^4 cells per ml at the time of inoculation to a maximum of 1.4×10^6 cells per ml. As in experiments for which the protozoa population was present initially in the system, the maximum recorded protozoan density occurred when nearly 50 per cent of the total secondary oxygen uptake had been exerted. The large oxygen uptake in flask B after the introduction of the protozoa seed could not be attributed to carryover of cells and/or substrate, since an insignificant oxygen uptake was observed in the protozoa inoculum control.

A parallel study was conducted at low substrate concentrations in the BOD bottle. The time of introduction of the protozoa-rich seed



Protozoa Count, Flask B	
Time, hr.	Protozoa/ml
24	1.3×10^4
43	1.4×10^6
61	6.7×10^3
139	No Motility

Fig. 10 - WARBURG STUDY USING SMALL INOCULUM OF ACCLIMATED PASTEURIZED SEED.

is indicated by the arrow on Figure 11. System B was inoculated with 1 ml of a 1/10 dilution of the protozoa inoculum used for system A. In systems A and B a second stage of oxygen uptake was observed; the second stage in system B lagged that in system A. In like manner the growth of protozoa in system B lagged that in system A. It is interesting to note that the maximum protozoan density attained in either system was much less (in the order of 1000 cells per ml) as compared to other experiments at low substrate concentrations (Figures 5, 6 and 7). However, the protozoa in these systems consisted almost entirely of ciliates of the type Enchelys. These protozoa are fairly large (25-50 μ), and this may account for the somewhat lower protozoan density than that which is usually observed at these substrate concentrations.

Influence of Predation on Other Parameters of Biochemical Response

In attempting to arrive at sound overall or general theory for the exertion of biochemical oxygen demand, it was of interest to study the effect of the presence of predator organisms on such biochemical response parameters as the substrate removal, biological solids production, and changes in protein content as well as oxygen uptake. These aspects were investigated in long term Warburg studies. Figure 12 shows changes in biochemical parameters for a normal sewage seed which contained protozoa, while Figure 13 shows results using a pasteurized sewage seed which was devoid of protozoan activity. In Figure 12 it is seen that glucose, as measured by the anthrone test, was exhausted from the system slightly before the occurrence of the peak in biological solids. Also a considerable rise in anthrone reactive material was noticed after 56 hours of aeration. This rise occurred during the second stage of oxygen

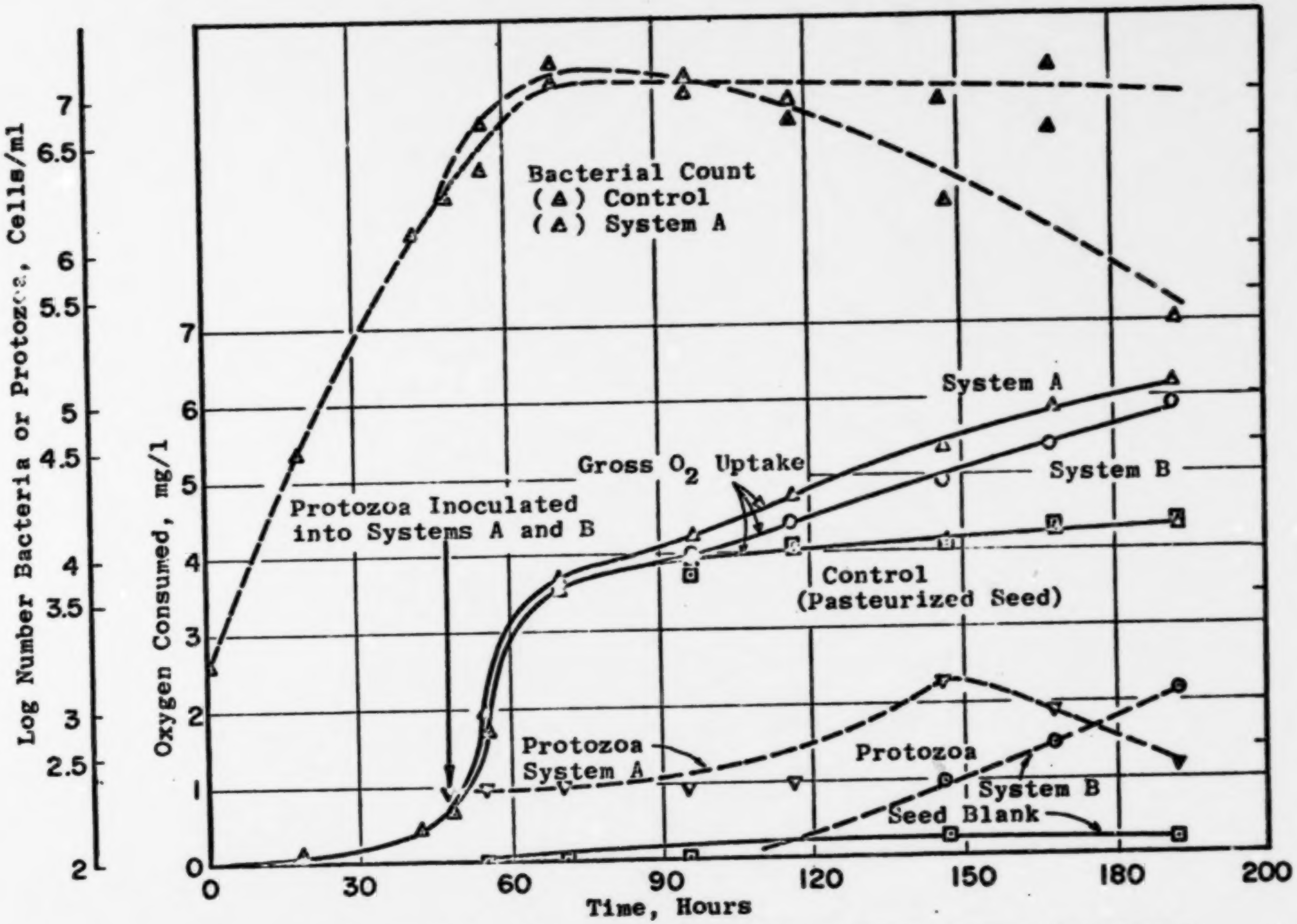


Fig. 11 - BOD EXERTION, BACTERIA AND PROTOZOA COUNTS IN THE BOD BOTTLE, USING 8 mg/1 GLUCOSE.

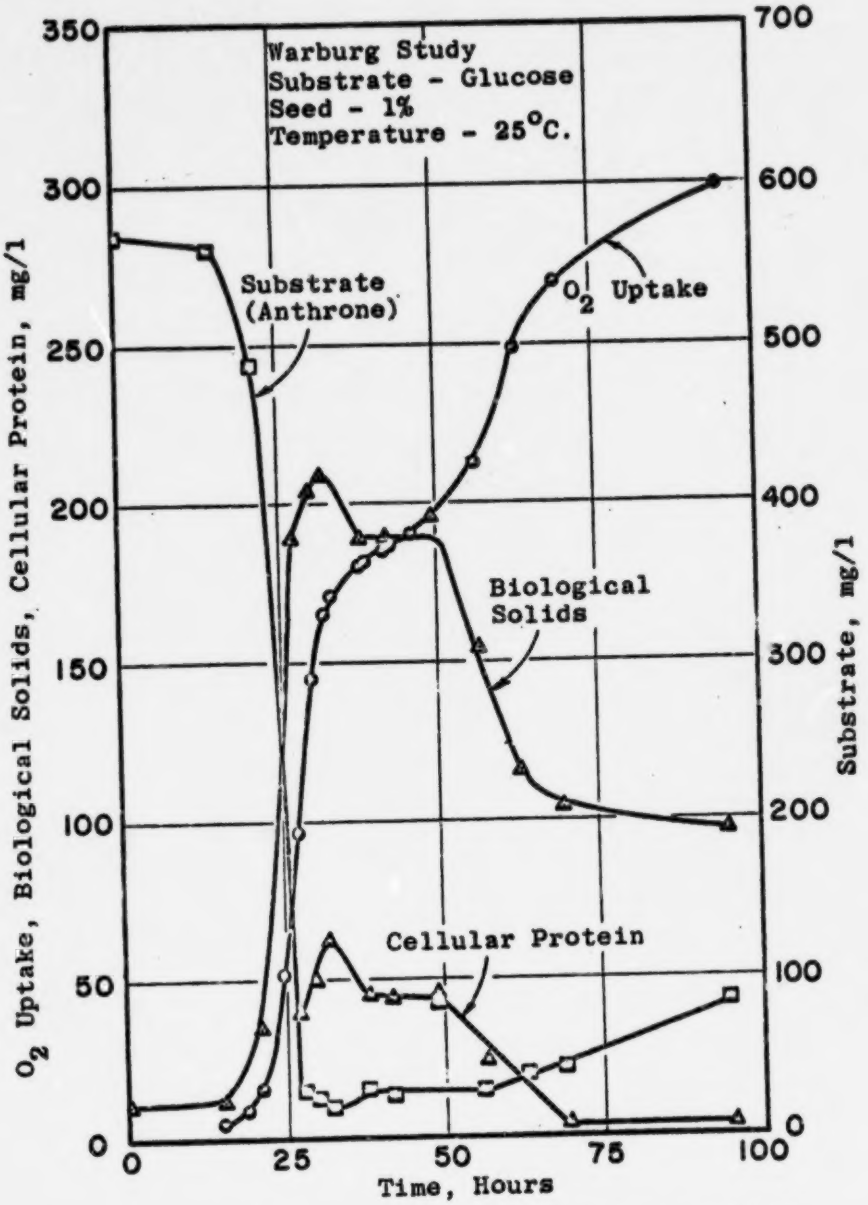


Fig. 12 - LONG TERM AERATION STUDY USING SEWAGE SEED.

uptake. Maximum biological solids and protein concentration occurred simultaneously at the start of the break in oxygen uptake. Both of these parameters decreased slightly before initiation of the second stage of oxygen utilization. During the pause or plateau in oxygen uptake, biological solids and protein content in the system remained stationary, and both of these parameters decreased during the second stage of oxygen uptake.

The results shown in Figure 13 were obtained using a pasteurized sewage seed. Glucose removal occurred slightly before the peak in biological solids concentration, as in the previous experiment; however, in Figure 13 there was no rise in anthrone reactive material during the latter part of the study. Also, the oxygen demand was exerted in a single phase. The peaks in biological solids and protein concentration again occurred close to the time of the break in the oxygen uptake and substrate removal curves. However, the decrease in biological solids and protein content after the removal of exogenous substrates was much less than for the system shown in Figure 12.

The Effect of Protozoa on the "Endogenous" Oxygen Uptake of a Pure Bacterial Culture

In a previous report the authors have criticized the use of the term "endogenous" respiration to describe, for heterogeneous populations or activated sludge, that portion of the total system response which occurs after the removal of exogenous substrate since, if the second stage of oxygen uptake is attributable to protozoa, it would involve their metabolism of the bacterial population, which to the protozoa represents an exogenous substrate.

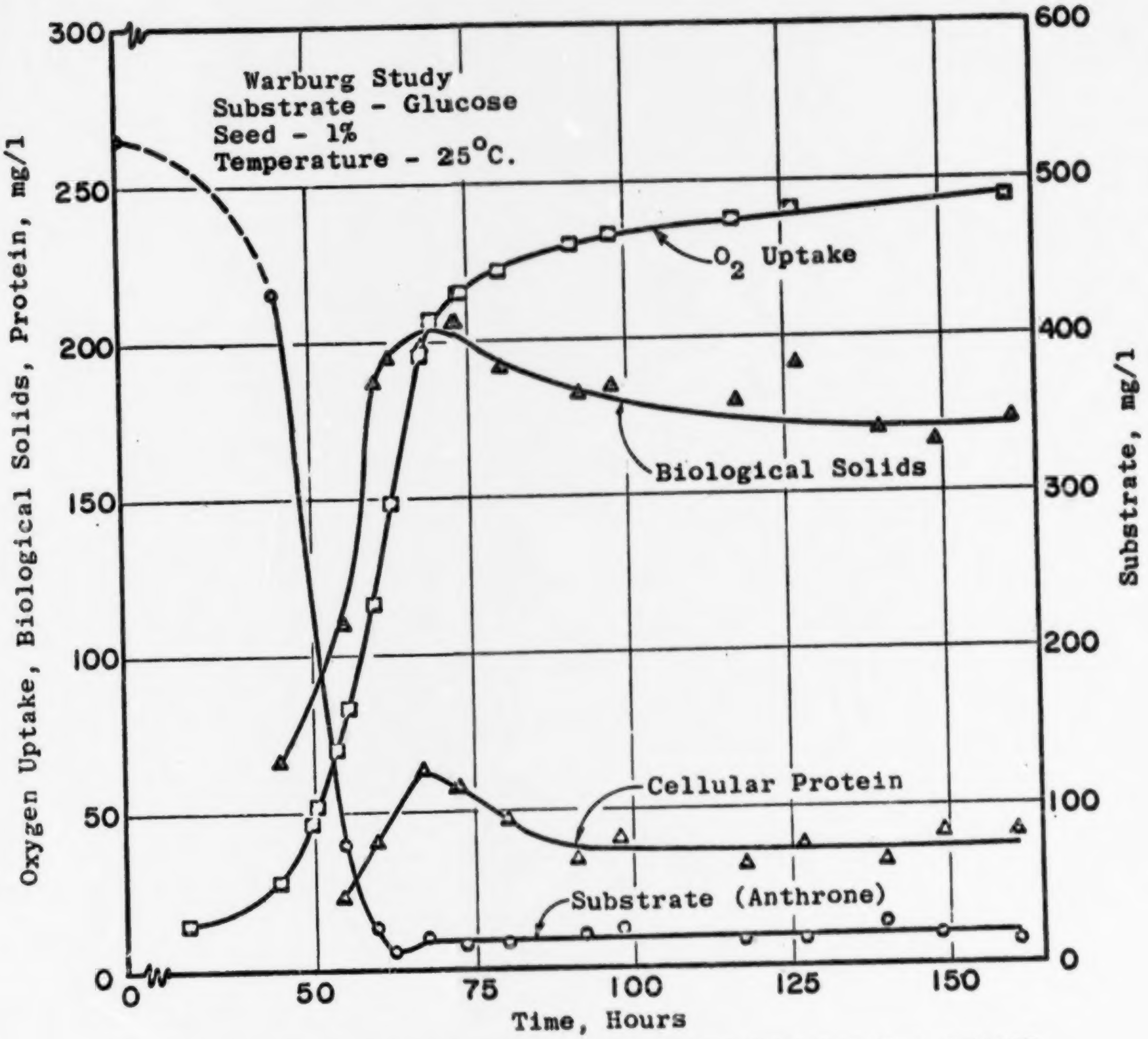
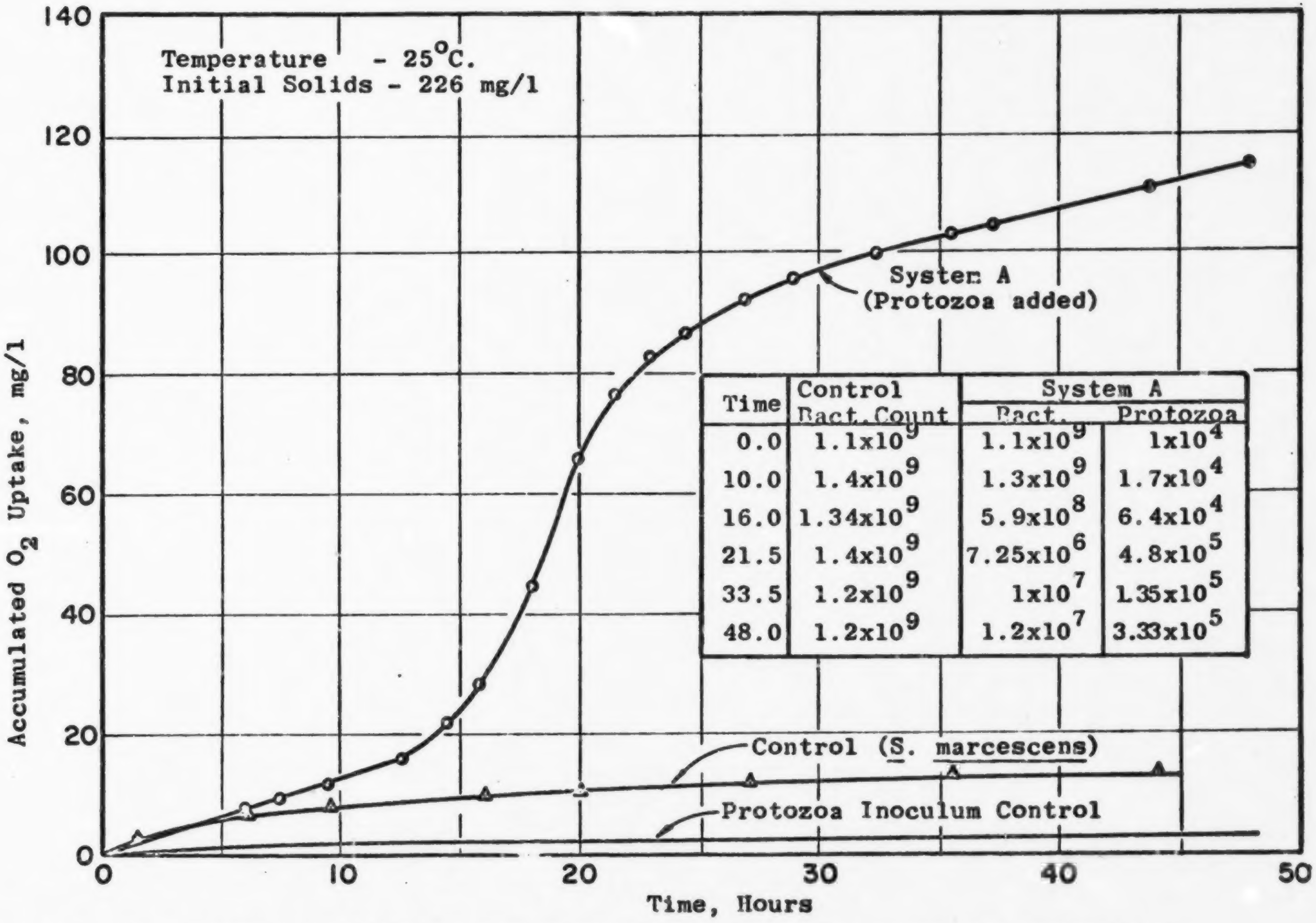


Fig. 13 - LONG TERM AERATION STUDY USING PASTEURIZED SEWAGE SEED.

In order to show the difference between oxygen uptake during endogenous metabolism of the bacteria and the so-called endogenous metabolism in a heterogeneous population and to gain information concerning the contribution made by the protozoa to the second stage of oxygen uptake, Warburg studies were made in which protozoa were inoculated into a pure culture suspension of bacteria devoid of substrate. In these experiments a washed suspension of *Serratia marcescens* was used. Figure 14 shows oxygen uptake for a system into which protozoa were inoculated, and for the *Serratia marcescens* and protozoa inoculum controls. Bacterial and protozoan counts are also given. The initial bacteria count due to carryover in the protozoa inoculum was 2.9×10^6 cells per ml; i.e., the bacterial carryover was negligible in comparison with the original number of bacteria (*S. marcescens* at 1.1×10^9/ml). The oxygen consumption by the protozoa inoculum control was quite low. Comparison of the results for these systems leaves little doubt as to the contribution of the protozoa to the second stage of oxygen uptake. These data also throw considerable doubt upon the normal concept of endogenous respiration as applied to heterogeneous populations containing protozoa.

DISCUSSION

The results of this study provide definite experimental proof of the validity of Theory 1. More specifically, the plateau in the BOD curve represents the transition from a system governed almost entirely by bacterial metabolism to one governed almost entirely by the metabolism of the protozoa. This has been shown to be true not only for the BOD curve (dilute systems) but in



Time	Control	System A	
	Bact. Count	Bact.	Protozoa
0.0	1.1x10^{9}	1.1x10^{9}	1x10^{4}
10.0	1.4x10^{9}	1.3x10^{9}	1.7x10^{4}
16.0	1.34x10^{9}	5.9x10^{8}	6.4x10^{4}
21.5	1.4x10^{9}	7.25x10^{6}	4.8x10^{5}
33.5	1.2x10^{9}	1x10^{7}	1.35x10^{5}
48.0	1.2x10^{9}	1.2x10^{7}	3.33x10^{5}

Fig. 14 - WARBURG STUDIES ON THE INTERACTION OF A PURE BACTERIAL CULTURE AND PROTOZOA.

high energy systems as well, using heterogeneous populations. The results as shown in Figure 1 indicate the low probability for the functioning of Theory 3 in heterogeneous populations. In a previous report the authors have concluded that the mechanisms embodied in Theories 2 and 4 were not of general occurrence and, therefore, could not be cited as general explanations for the plateau in heterogeneous populations[11]. In addition, since chloramphenicol completely prevented bacterial growth immediately after its introduction, the nature of the results shown in Figure 1 lead to the conclusion that a secondary predominance in bacterial population (part of Theory 1) cannot be cited as a general cause of the plateau or the second stage of oxygen uptake in heterogeneous populations. It is concluded that the predominant cause for the second stage of O_2 uptake described by the carbonaceous BOD curve is not the endogenous metabolism of the bacteria, but the growth of protozoa using bacterial cells as exogenous substrate. These conclusions are in contradiction to concepts prevalently held in the field regarding the role of protozoa in the exertion of biochemical oxygen demand.

Based upon these results, it is now possible to present a more theoretically sound and complete concept of the mechanism of BOD exertion for heterogeneous populations: In a typical oxygen uptake curve with two phases separated by a well-defined pause or plateau, the first phase (the more rapid) is associated almost exclusively with bacterial replication due to metabolism of the extracellular carbon source. During this rapid phase of growth, the cells respire a part of the substrate to provide energy for anabolic or synthetic processes. The bacterial population increases and finally reaches a limiting value depending upon the environmental conditions and the amount of available substrate. Following the exhaustion of exogenous substrate, the bacteria begin to oxidize the readily available intracellular substances, e.g., carbohydrates and protein. Up to this point the contribution to oxygen uptake by protozoan activity is negligible. The primary protozoan food consists of bacteria. The possibility of direct utilization of a soluble carbon source by the protozoa cannot be overlooked[4, 22], but with a heterogeneous population and a relatively dilute soluble substrate, protozoa which use this mode of metabolism are at a distinct disadvantage. Thus, the experimental results indicate that the first phase of oxygen uptake (which corresponds to exhaustion of exogenous substrate) is brought about almost exclusively by the metabolism of the bacteria. However, based upon the results presented herein, Busch's statements[2] concerning the second phase of oxygen uptake, "The latter phase involves both the oxidative organisms and predatory organisms," and "Any subsequent utilization of oxygen in excess of the endogenous rate is due to predator activity," require considerable clarification. The plateau cannot be considered simply as a pause between two phases of oxygen uptake but is rather the second of three distinct phases. The so-called plateau or pause between the two stages of O_2 uptake actually represents the endogenous bacterial metabolism which, if continued in the absence of protozoa, would add relatively little to the accumulated oxygen uptake. During this period the bacterial count lingers in the range of maximum population density. After some time, depending upon the type and amount of protozoa present in the system, their increasing multiplication is manifested as a noticeable rise in oxygen uptake. Since this third stage is caused almost entirely by the growth of protozoa feeding on the bacteria, the bacterial density rapidly declines, decreasing even further the contribution of endogenous bacterial metabolism. The third phase of O_2 uptake is usually expressed as an autocatalytic curve due to the growth of protozoa from initially small numbers; this statement is borne out by the results shown in Figures 10, 11, and 14. In the general case nearly half of the third stage O_2 uptake is expressed by the time the protozoan population density attains its maximum value. The remaining half of the third stage curve is expressed by decreasing rate kinetics and corresponds to a slowly declining protozoan population.

Since the oxygen uptake curve described by a heterogeneous population in a discontinuous (batch) system is a summation of the oxygen utilization curves produced by the bacteria, in sequence with the O_2 uptake curve due to protozoan activity, the length of the "plateau" or the duration of endogenous bacterial metabolism is a function of the rate of growth and the time required for each type of population to reach its maximum density. A long "plateau" is created in the oxygen uptake curve when the protozoan growth lags considerably the bacterial growth, i.e., when the occurrence of bacterial and protozoan maximum counts are very far apart on the time scale (Figures 5 and 6). A shorter pause is generated when the peaks in the two populations occur at shorter time intervals. Since the protozoa-bacteria ratio in the initial seed is one of the factors contributing to the spacing of attainment of maximum bacterial and protozoan populations, the authors are in agreement with Busch's view that the protozoa-bacteria ratio can mask or "blur" the plateau.

While it is herein stated, based upon the experimental results, that a substantial proportion of oxygen uptake during the exertion of biochemical oxygen demand is directly due to oxygen utilization by the protozoa while feeding on the bacteria as substrate, other works reported in the literature do not agree with this conclusion. Protozoa have been regarded as enhancing purification (measured as oxygen uptake) by aiding in the continuous lowering of the bacterial population below some limiting number, thereby stimulating further bacterial multiplication which is responsible for the intensified or continued purification or oxygen uptake. This theory portraying the role of protozoa during the exertion of biochemical oxygen demand was proposed by Butterfield et al.[5] and was shared by Phelps[23], Zehnpfennig et al.[6] and Javornicky et al.[7]. These investigators regarded protozoa as having an indirect rather than a direct function in oxygen utilization; increased oxygen uptake (more purification) caused by the addition of predators was considered to be due to the physiological activities of the bacteria rather than the protozoa. This interpretation, first made by Butterfield et al.[5] was based on results obtained in dilute systems (the BOD bottle) in which oxygen uptake was the sole criterion for measuring purification; higher oxygen uptake meant greater purification. According to Butterfield et al.[5] substrate removal took place during the entire course of oxygen uptake, and the continuous destruction of bacteria by the predators maintained an optimum ratio of bacteria to food. Phelps[23] did not present any experimental data; however, he accepted this mode of action for protozoa in the purification process. The hypothesis of Butterfield and his co-workers apparently misguided Zehnpfennig et al.[6] when they envisioned the BOD reaction as occurring in two stages. According to them, the first phase of oxygen uptake was controlled by the substrate concentration and proceeded according to first order reaction kinetics. They felt that zero order kinetics would prevail in the second stage of oxygen uptake since it was thought that oxygen uptake in this stage depended upon the reproduction rate of the bacteria which were continuously multiplying while being continuously destroyed by the protozoa. Javornicky and Prokesova[7] agreed with the theory of Butterfield, since they obtained somewhat similar experimental results. However, the data presented by these authors cannot be used to test Butterfield's theory, since the only parameters measured were oxygen uptake and bacterial and protozoan density; thus there was no way of knowing if exogenous substrates were present in the system throughout the entire course of oxygen uptake.

Based upon the following findings, it is concluded that the concept concerning the role of protozoa in the exertion of biochemical oxygen demand herein presented supplants that of Butterfield et al.:

1) Substrate removal (see Figures 6 and 12) occurred before the beginning of the second stage of oxygen uptake, and no third stage of oxygen uptake was observed in the absence of predator activity. The removal of substrate by the end of the first phase of oxygen uptake has been shown by Grady and Busch[24], McWhorter and Heukelekian[4], and by the authors[11]. With the complete absence of exogenous substrate after the first stage of oxygen uptake, the probability of further bacterial multiplication stimulated by predator activity is very low. Hence, the increased purification or stabilization (measured as oxygen uptake) due to the presence

of protozoa must be directly ascribed to the respiration of the protozoa feeding on the bacterial population.

2) The oxygen uptake curves shown in Figure 14 and the decrease in turbidity brought about in response to the addition of protozoa to the *Serratia marcescens* cell suspension also indicate a direct contribution to oxygen uptake in the last stage by the protozoa.

3) Chloramphenicol, which completely arrested bacterial replication before the start of the third stage, did not inhibit the oxygen uptake during the third stage.

These findings lead to the conclusion that the protozoa do not function simply as agents which help intensify waste water purification by the bacteria, but play an independent role in completing the purification process. In a discontinuous system, purification is a phasic phenomenon; the first phase of BOD exertion is accomplished mainly by bacteria, and the second phase consists mainly of the destruction of this biomass (clarification) and is accomplished through the metabolic activity of the predators. The contribution of endogenous metabolism by the bacteria (the actual second stage) is negligible and can be eliminated from consideration as a factor in BOD exertion. The role of protozoa in clarifying activated sludge effluents has been recognized by many workers[21, 25, 26, 27]. It must now be realized that this clarification, whether it occurs in the activated sludge tank or in the BOD bottle, is accompanied by a considerable oxygen demand. If waste stabilization is to be measured by a biochemical oxygen demand test, the energy required for this particular phase of purification, which fortunately occurs in the usual case after the bacteria have removed the original organic pollutant, must be either accounted for or disregarded in assessing the pollutional strength of a waste water. However, if the latter alternative is followed, the uses to which the BOD test can be put must be restricted and this would require considerable revision of the technical philosophy of measuring the strength of a pollutant, by measuring the amount of oxygen required by the biota to "stabilize" it. If the five-day BOD test is used, it is seen by the results of the present study that the respiratory activities of the protozoa can contribute significantly to the amount of carbonaceous BOD registered. It has been shown here that under normal seeding conditions protozoan activity usually contributes approximately thirty per cent of the five-day BOD. It would therefore seem apparent that the BOD test cannot be interpreted solely in terms of bacterial respiration or as a test for substrate removal. Furthermore, there is no simple kinetic model for the BOD reaction since it is a complex of two growth cycles (bacterial and protozoan) which normally occur in series and are sufficiently displaced to cause a "plateau." It is also important to note that the amount of oxygen uptake exerted during the second stage depends, apart from the quantity of food (the bacterial cells), upon the type of protozoa present. In the present studies it was noted that the small flagellated protozoa which were passed by the five μ filter were not particularly effective in exerting oxygen uptake. However, most of the protozoa responsible for the second stage of oxygen uptake passed a ten μ filter.

In closing, it is emphasized that the results of this study showing the dominant role of protozoa in causing oxygen uptake beyond the plateau during exertion of carbonaceous BOD and the spacing of the sequential growth cycles for the bacteria and protozoa which actually allows the observation of the "plateau" (constituting proof of Theory 1) provides a sound explanation for diphasic oxygen uptake separated by a plateau only for heterogeneous populations. While such populations are the ones of primary interest in the pollution control field, plateaus have been found to occur in pure culture studies as well. On the basis of over three years of concentrated research effort, the authors have been able to conclude that Theories 2, 3 and 4 are the least likely explanations for the plateau in heterogeneous populations; however, this does not eliminate the validity of these theories. This work has led to the conclusion that in the general case (which must be accorded prime consideration in engineering design and process control) when the seeding material contains both bacteria and protozoa the plateau will be generated in accordance with Theory 1 as herein delineated.

CONCLUSIONS

1. In both high and low energy systems employing heterogeneous populations the plateau is brought about in the general case by the lag between sequential growths of the bacterial population metabolizing the exogenous substrate and the predator population metabolizing the bacterial population produced in the first stage of oxygen uptake.

2. The plateau actually represents the endogenous respiration phase of bacterial metabolism which, if no protozoa were present, would be manifested as a second stage of low magnitude. The secondary rise in oxygen uptake (second stage) is actually a third stage of oxygen uptake caused by the predator population.

3. The relative protozoa-bacteria density in the initial inoculum can determine whether a plateau will be observed.

4. The length of the plateau may be considered proportional to the relative time lag between the peaks in bacterial and protozoan populations.

5. Former concepts which maintained that the excess "purification" (i.e., oxygen uptake) attained in the presence of protozoa was brought about by continued bacterial growth stimulated by predator activity are supplanted by the results of this study. It has been shown that the increased oxygen uptake is caused directly by the protozoa in metabolizing the bacterial cells; thus the protozoa play an autonomous role in oxygen uptake in addition to their well-known function of clarifying the liquor. Under normal seeding conditions, during a five-day BOD test, protozoa are responsible for approximately thirty per cent of the BOD exerted.

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PAPER 13

EFFECT OF SLUDGE CONCENTRATION ON VARIOUS ASPECTS OF BIOLOGICAL ACTIVITY IN ACTIVATED SLUDGE

By

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EFFECT OF SLUDGE CONCENTRATION ON VARIOUS ASPECTS OF BIOLOGICAL ACTIVITY IN ACTIVATED SLUDGE

By

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There has been continued interest in the formulation of relationships between sludge growth and substrate removal rate, or purification rate, for activated sludge systems. There has also been great interest in the prediction of sludge yields. One of the first definitive studies concerning these aspects was reported by Ruchhoft, et al.(1), who found a simple linear relationship between the rate of substrate removal and initial sludge concentration using glucose as the sole carbon source. Eckenfelder and McCabe(2) also indicated a proportional increase in the rate of substrate removal with increasing biological solids concentration for pharmaceutical and brewery wastes. Wuhrman(3) has shown a similar relationship for such compounds as glucose, lactate and peptone. Comparison of Wuhrman's data on glucose with those of Ruchhoft, et al. indicates fairly good agreement and yields the impression that the slope of a plot of substrate removal rate vs initial biological solids concentration is dependent primarily upon the type of substrate employed; i.e., there is a linear relationship for each type of waste. Although it would be ideal from an engineering standpoint if such a relationship were entirely substrate-dependent, there is no published data (to our knowledge) which indicate that the relationship remains constant over a prolonged period of operation.

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Many studies on carbohydrate wastes have been concerned primarily with the prediction of the sludge yield. Placak and Ruchhoft(4) reported sludge yields on carbohydrate wastes in the range of 65 to 85%. Hoover, et al.(5) studied the assimilation of dairy waste using COD as a parameter for measuring substrate removal, and they found that approximately two-thirds of the carbon source was channelled into synthesis during short-term experiments. Sawyer(6) has tabulated data from many sources and has concluded that sludge yields in the range of 50 to 60% can be expected; for glucose he has indicated a range of 44 to 64%. Gaudy and Engelbrecht(7) obtained a cell yield of 60%, using glucose as substrate. McKinney(8) has concluded that two-thirds of the ultimate oxygen demand of the organic matter being metabolized is converted into cellular mass. Recently McWhorter and Heukelekian(9) have reported on studies employing glucose as substrate wherein cell yields from 28 to 35% were obtained; these workers used COD as the parameter of substrate measurement. McCarty(10) has reported that for glucose 0.55 grams of dry sludge solids will be produced per gram of COD removed. Servizi and Bogan(11) have discussed the relationship between the free energy content, ATP yield, and cell yield. Their assumption that the yield of ATP is related to the free energy content of the exogenous substrate may be subject to considerable debate, since the ATP yield is directly related to the biochemical pathway by which the compound is metabolized. However, they ultimately related cell yield to COD, and in cases where the COD of the waste largely represents biologically available substrate, this latter relationship does appear to be valid just as such a relationship involving BOD or carbon content would similarly be valid. For carbohydrate wastes they indicated that 0.38 grams of cells or sludge would be produced per gram of COD removed.

It can be seen from the brief review above that even for a relatively simple carbohydrate, such as glucose, considerable ranges of cell yields have been reported. It might be concluded from such a review that either the energy concept of Servizi and Bogan is not entirely correct, or that differences in experimental procedure have led to discrepancies or variation in the cell yields. It would also seem from a review of the literature that most of the work which has been reported is based on relatively short-term experiments, and that a large proportion of the work dealing with cell yields has involved the measurement of relatively few experimental parameters. It was felt that a considerable insight into the constancy of the cell yield and the relation between substrate removal rate and initial solids concentration could be obtained by conducting an integrated, long-term study under highly controlled operational conditions using only one carbon source.

MATERIALS AND METHODS

Three batch activated sludge systems were developed, from which sludge was harvested for the studies herein reported. Each was operated for approximately ninety days. These units are designated as follows:

Unit A - a 15-liter system fed glucose at a concentration of 5000 mg/l/day. Initial seed population was taken from the primary clarifier effluent of the Stillwater municipal treatment plant.

Unit B - a 15-liter system fed glucose at a concentration of 1000 mg/l/day. Initial seed population was obtained from the primary clarifier effluent of the Stillwater municipal treatment plant.

Unit C - a 1.5-liter system fed glucose at a concentration of 5000 mg/l/day. Initial seed population was obtained from Unit B.

The composition of the synthetic waste for units A and C is given in Table I. For unit B, all concentrations were 1/5 those given in the table. The units were fed once daily in accordance with the following procedure: After 23 hours of aeration, a third of the mixed liquor was wasted and the remainder was settled for one hour. One-half of the remaining volume was wasted as supernatant, and the aeration liquor was made up to the original volume, with all waste constituents added to yield the concentrations given in Table I.

At various times the sludge wasted from these units was harvested and used for separate batch experiments to assess the effect of initial biological solids concentration upon specific parameters of biological activity. In all cases, the sludge was washed twice with 0.05 M phosphate buffer. Portions of the washed sludge were suspended in inorganic salts solution (see Table I) to yield systems of varying initial biological solids concentration. In general three to five different initial sludge concentrations were made up to 1.5 liters in large tubes used for batch aerators. Forty ml of each suspension were then withdrawn to be used for measuring endogenous O_2 uptake on the Warburg apparatus. Glucose was added to all units at either 1000 or 2000 mg/l. The inorganic salts concentration used at the 1000 mg/l level was the same as that used for unit B; at the 2000 mg/l level salts concentrations were doubled. Immediately after the carbon source had been added, a 40-ml portion was withdrawn from each unit and placed on the Warburg respirometer. Samples were also withdrawn for measurement of biological solids concentration, using the membrane filter technique(12) and COD(12), and glucose (13) concentrations in the membrane filtrate.

Throughout these studies the Warburg apparatus was run at 25 ± 0.1°C. at an oscillation rate of 100 strokes/min. Units A, B and C as well as the 1.5-liter units used in the experiments were operated in a room in which temperature was controlled at 25 ± 0.5°C. and the temperature of the aeration liquor was 23.5 ± 0.5°C. Compressed air was supplied in excess by means of coarse carborundum diffuser stones. Electrometric dissolved oxygen measurement in the control units during the period of substrate removal indicated no less than 90% saturation. The pH remained constant at 6.7 ± 0.1.

TABLE I

COMPOSITION OF SYNTHETIC WASTE

Constituent	Concentration
Glucose	5000 mg/l
$(NH_4)_2SO_4$	2500 "
$MgSO_4.7H_2O$	500 "
$FeCl_3.6H_2O$	2.5 "
$MnSO_4.H_2O$	50 "
$CaCl_2.2H_2O$	37.5 "
Tap Water	100 ml/l
1.0 M Phosphate Buffer, pH 6.8	60 "

RESULTS

A considerable amount of data was obtained for continuously operated units A, B and C, but will not be reported here. The importance of these units for the studies herein reported is that they served as a source of activated sludge grown under constant conditions for a prolonged period of time. During this study 15 experiments were conducted on sludge harvested from unit A, 17 on sludge from unit B, and 12 on sludge from unit C. Results of one such experiment, showing the parameters examined, are presented in Figure 1. It is seen that substrate removal as well as sludge growth can be plotted in accordance with zero order kinetics. This was typical of all 44 experiments. The time of maximum solids production corresponded closely to the time of glucose and COD removal, and glucose was removed at a slightly greater rate than the total COD. Since glucose was the only organic substrate in the medium, the divergence of the two curves is attributed to the liberation of metabolic intermediates or end products during glucose metabolism. In general, intermediates were released in all experiments using sludge developed at the 5000 mg/l glucose level (units A and C), and at times in unit B, but to a lesser extent. For the experiment shown, oxygen uptake was also linear; this occurred in most experiments, but was not typical of all. At fairly low initial solids concentrations a typical "S shaped" curve was obtained, but solids production and substrate removal remained essentially linear. The percentage of the theoretical oxygen demand exerted at the time of COD removal was 13, while at the time of glucose removal it was 10. Results of all experiments are summarized in Tables IIA (unit A), IIB (unit B), and IIC (unit C), with accompanying explanations of the various columns in the table.

In Figure 2 changes in biological solids and COD concentrations are compared for three experiments in which various initial solids concentrations of an identical sludge sample were employed. The COD removal rate and sludge growth rate for each system are also given in the figure. The rate constants were calculated during the linear phase as substrate removed per hour, or biological solids increase per hour. It can be seen that the sludge growth rate and COD removal rate approximately doubled as initial solids concentration was doubled.

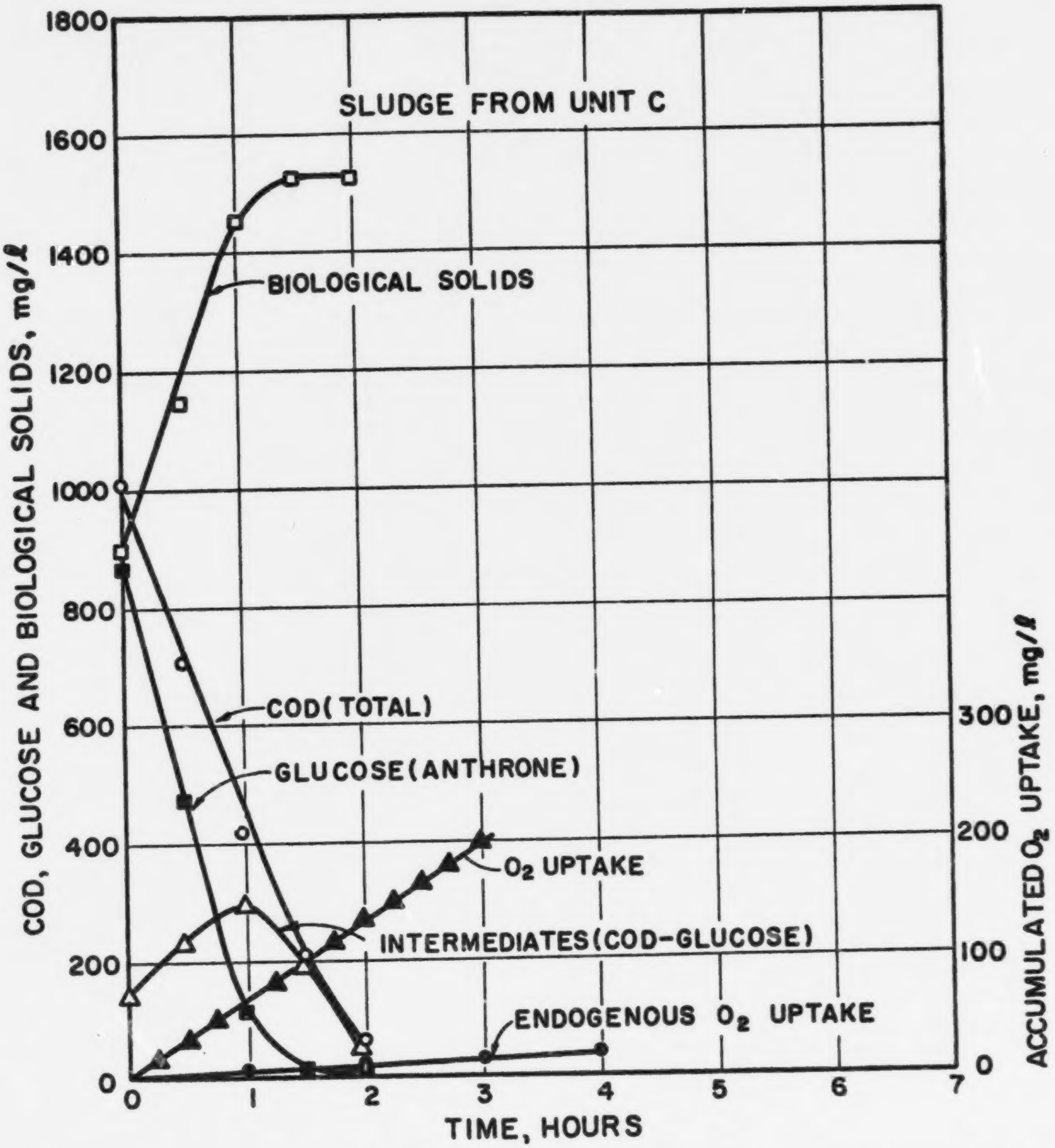

FIG. 1 - LINEAR SUBSTRATE REMOVAL, RESPIRATION AND SYNTHESIS IN A TYPICAL EXPERIMENT.

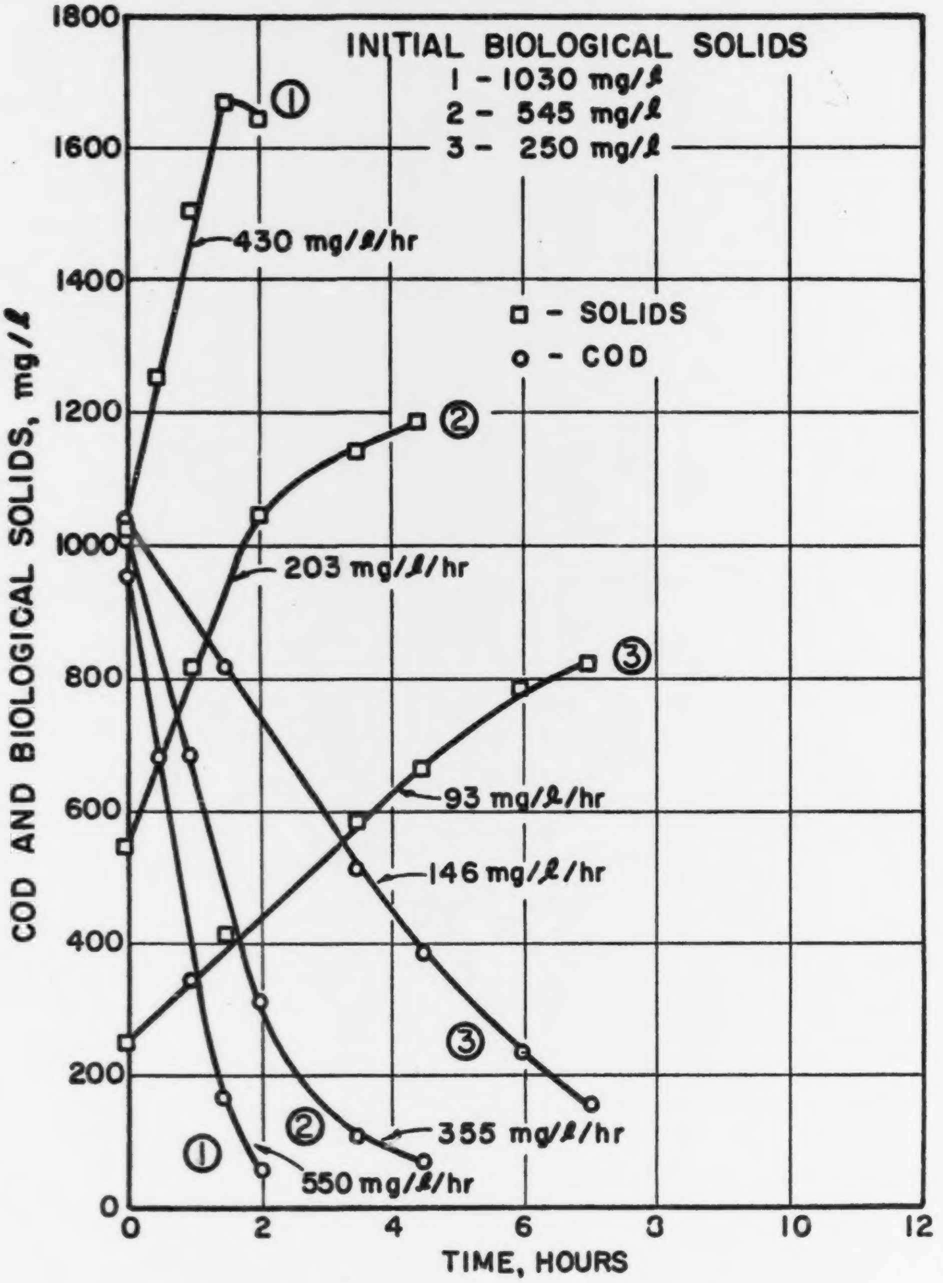

FIG. 2 - EFFECT OF INITIAL BIOLOGICAL SOLIDS CONCENTRATION ON THE RATE OF SUBSTRATE REMOVAL AND SLUDGE GROWTH (SLUDGE DEVELOPED IN UNIT C).

This type of result was typical for all experiments performed using identical sludge samples.

Effect of Initial Biological Solids Concentration on Rate of Substrate Removal

Unit A

At various times during the three months of operation of unit A sludge was harvested for conducting studies of the type illustrated in Figure 2. The group numbers shown in Table IIA indicate experiments accomplished using portions of a single sample of sludge. Four such groups of experiments were performed, using sludge from unit A. In Figure 3 the COD removal rate (mg/l/hr) is plotted against initial biological solids concentration for each group of experiments. These rates are given in Table IIA, column 5. It can be seen that a simple, straight line relationship is obtained for each group (identical sludges), but there is no one relationship which describes quantitatively the effect of increasing biological solids concentration for the entire three months' period of operation; i.e., a single straight line relationship did not exist for the sludge grown in unit A even though the experimental conditions under which the sludge was grown remained constant.

Microscopic examinations of the sludge in the control unit were made throughout the study. It is interesting to note that the variation in the relationship between COD removal rate and biological solids concentration could be correlated with gross changes in biological predominance in the unit. Experiments in group I were made after 40 days of operation of the unit, at which time the color of the sludge was golden-brown. The population consisted of the usual bacterial floc, and many free-swimming protozoa were noted. Experiments in Group II were conducted after 50 days of operation, at which time the color of the sludge had changed to dark brown. No drastic morphological changes had occurred, but there was a slight increase in filamentous forms. It is seen that the slope of the line was considerably less than that for Group 1. The sludge in unit A continued to darken, and turned from brown to black. The color could be attributed to the pigment of the organisms, since the membrane filtrate was essentially colorless. Bacterial floc was again present, but chain forms predominated. At this time (60 days)

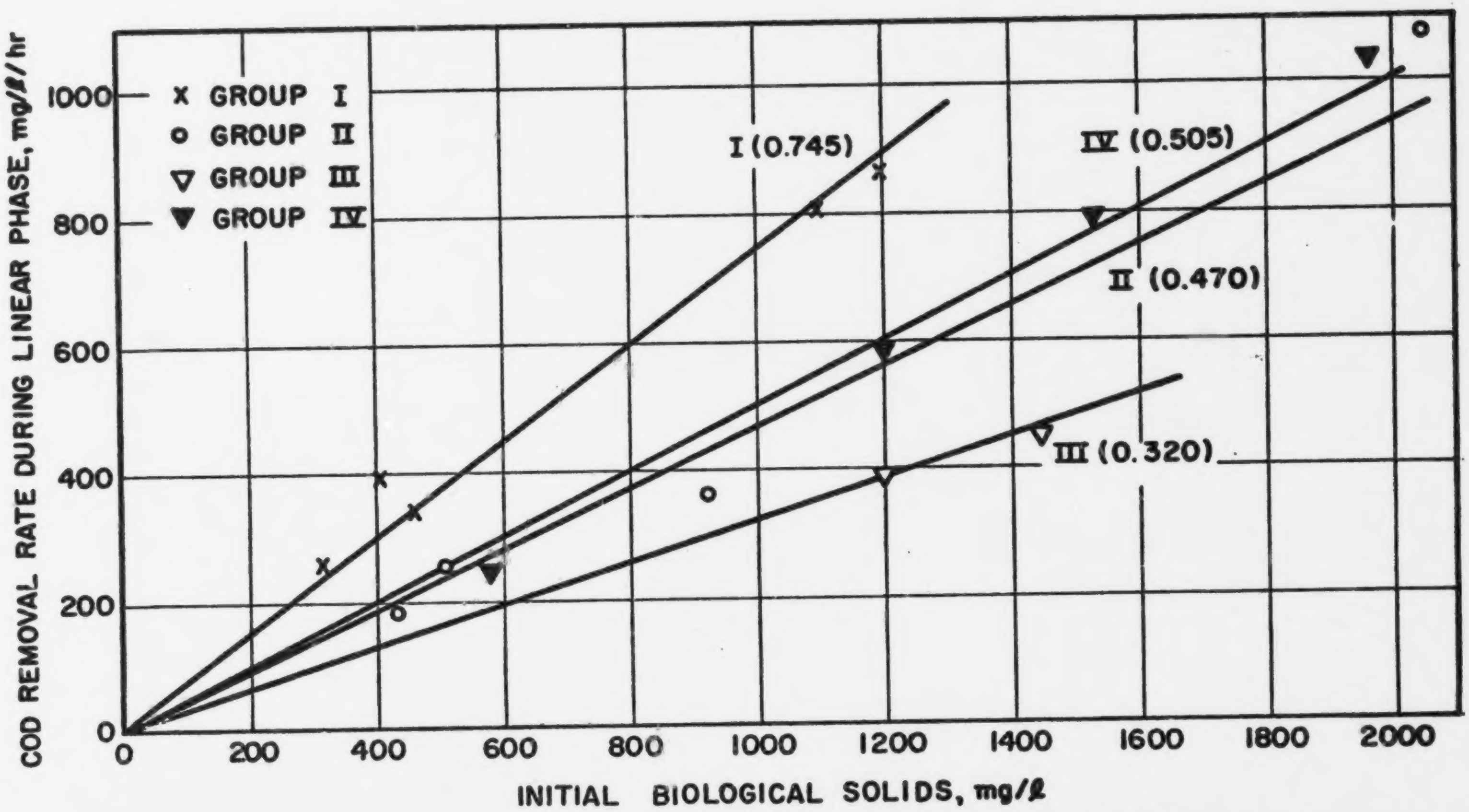

FIG. 3 - EFFECT OF INITIAL BIOLOGICAL SOLIDS CONCENTRATION ON THE RATE OF SUBSTRATE REMOVAL; SLUDGE TAKEN FROM UNIT A AT VARIOUS TIMES DURING A 90 DAY PERIOD OF OPERATION.

TABLE II (A)

SYSTEM PARAMETERS FOR STUDIES USING SLUDGE HARVESTED FROM BATCH UNIT A

1	2	3	4	5	6	7	8	9	10	11	12	13	14
Group No.	Initial Biolog. Solids mg/1	Initial COD mg/1	Endogenous Respiration mgO_2/hr/gm Sludge	Rate COD Removal mg/1/hr	Rate of COD Removed mg/1/hr/mg/1 In. Solids	Rate of Sludge Production mg/1/hr/mg/1 In. Solids	Yield %	Yield %	Yield %	% Theoretical O_2 Uptake (COD)	% Theoretical O_2 Uptake (Glucose)	Percent Recovery	
												Material Balance	Energy Balance
I ×	316	2000	7.6	247	0.780	0.396	56	59	50.8	30.0 - 98	26.7 - 98	90	98
I ×	470	2140	8.2	341	0.725	0.339	50	53	46.8	29.2 - 97	20.3 - 98	82	90
I ×	410	2020	8.1	396	0.965	0.566	60	60	58.7	21.0 - 94	17.6 - 98	86	95
I ×	1100	1900	8.9	801	0.728	0.382	63	55	52.5	14.7 - 96	10.0 - 98	83	91
I ×	1200	1800	8.3	862	0.718	0.434	70	72	60.5	13.9 - 96	9.2 - 98	89	97
II ○	435	2020	11.5	189	0.435	0.398	78	88	91.5	27.8 - 95	24.2 - 98	112	123
II ○	926	1920	9.7	367	0.396	0.321	84	88	81.2	27.6 - 98	23.4 - 98	117	129
II ○	2050	1880	11.0	1078	0.526	0.317	68	67	60.4	16.0 - 98	8.8 - 98	90	98
II ○	515	2214	7.8	263	0.511	0.340	69	68	66.5	29.6 - 98	22.6 - 98	104	113
III ▽	1200	1960	14.6	394	0.328	0.238	80	76	72.5	20.4 - 84	20.4 - 98	110	120
III ▽	1450	1990	12.4	450	0.310	0.236	75	80	76.2	20.1 - 98	18.1 - 98	104	110
IV ▼	580	1800	8.7	247	0.425	0.302	70	71	71.0	21.7 - 76	21.7 - 98	103	112
IV ▼	1200	1760	9.2	581	0.484	0.341	71	72	70.5	19.7 - 98	15.6 - 98	96	106
IV ▼	1967	1760	8.5	1035	0.526	0.315	65	65	60.0	14.0 - 98	10.0 - 98	84	93
IV ▼	1532	1760	8.6	791	0.516	0.314	66	66	61.0	16.0 - 98	12.0 - 98	87	96

TABLE II (B)

SYSTEM PARAMETERS FOR STUDIES USING SLUDGE HARVESTED FROM BATCH UNIT B

1	2	3	4	5	6	7	8	9	10	11	12	13	14
												Percent Recovery	
Group No.	Initial Biolog. Solids mg/1	Initial COD mg/1	Endogenous Respiration mgO_2/hr/gm Sludge	Rate COD Removal mg/1/hr	Rate of COD Removed mg/1/hr/mg/1 In. Solids	Rate of Sludge Production mg/1/hr/mg/1 In. Solids	Yield %	Yield %	Yield %	% Theoretical O_2 Uptake (COD)	% Theoretical O_2 Uptake (Glucose)	Material Balance	Energy Balance
V •	228	1070	6.2	63	0.278	0.102	42	50	36.7	23.4 - 76	23.4 - 91	75	81
V •	432	1040	5.6	130	0.302	0.171	57	57	56.6	18.8 - 82	18.8 - 92	83	91
V •	558	1100	5.4	150	0.269	0.188	61	68	70.0	19.5 - 88	19.5 - 89	88	95
V •	1210	1040	6.2	450	0.372	0.268	69	73	72.0	16.3 - 93	16.3 - 98	91	100
VI ⊙	178	1100	8.1	57	0.321	0.167	50	53	52.0	14.1 - 51	14.1 - 77	81	88
VI ⊙	405	1060	6.1	129	0.318	0.190	63	63	59.8	20.8 - 89	20.8 - 90	91	99
VI ⊙	710	985	6.2	253	0.356	0.244	73	70	68.5	17.4 - 96	17.4 - 98	96	106
VI ⊙	1070	994	6.0	423	0.395	0.266	75	74	67.5	13.6 - 95	13.6 - 98	95	104
VII ●	318	996	7.2	139	0.437	0.303	61	65	69.4	18.6 - 95	18.6 - 98	85	93
VII ●	550	996	7.6	278	0.505	0.354	67	73	70.2	15.6 - 95	15.6 - 98	89	96
VII ●	932	990	6.2	511	0.548	0.384	70	70	70.1	12.1 - 98	12.1 - 98	87	95
VIII ○	345	1090	3.0	143	0.415	0.293	66	70	70.8	13.4 - 77	-	94	103
VIII ○	630	1060	2.4	265	0.420	0.330	73	77	78.6	14.6 - 80	14.6 - 83	96	106
VIII ○	970	1000	3.6	501	0.516	0.402	75	79	78.0	12.0 - 90	12.0 - 98	93	103
IX ⊕	298	1100	8.7	86	0.288	0.200	47	62	69.5	23.6 - 80	23.6 - 86	80	86
IX ⊕	640	1090	6.6	200	0.312	0.203	60	63	65.0	20.2 - 94	20.2 - 98	86	93
IX ⊕	1210	1020	6.7	431	0.356	0.198	53	57	55.7	15.3 - 95	15.3 - 98	72	80

TABLE II (C)

SYSTEM PARAMETERS FOR STUDIES USING SLUDGE HARVESTED FROM BATCH UNIT C

1	2	3	4	5	6	7	8	9	10	11	12	13	14
Group No.	Initial Biolog. Solids mg/1	Initial COD mg/1	Endogenous Respiration mgO_2/hr/gm Sludge	Rate COD Removal mg/1/hr	Rate of COD Removed mg/1/hr/mg/1 In. Solids	Rate of Sludge Production mg/1/hr/mg/1 In. Solids	Yield %	Yield %	Yield %	% Theoretical O_2 Uptake (COD)	% Theoretical O_2 Uptake (Glucose)	Percent Recovery Material Balance	Percent Recovery Energy Balance
X ■	560	1170	13.7	155	0.277	0.199	59	68	72.0	21.4 - 67	21.4 - 78	96	102
X ■	980	1090	14.2	275	0.281	0.169	59	65	60.2	16.5 - 73	16.5 - 87	86	94
X ■	1340	1145	14.5	430	0.321	0.194	62	58	60.5	12.2 - 73	12.2 - 92	79	86
XI ●	350	1130	6.5	207	0.590	0.296	57	55	50.2	28.3 - 82	28.3 - 98	96	103
XI ●	640	1100	4.9	358	0.560	0.242	52	48	43.2	22.7 - 84	22.7 - 98	83	90
XI ●	1340	1050	5.6	725	0.541	0.268	58	53	49.6	14.8 - 98	10.9 - 98	77	85
XII ▲	240	1060	7.2	138	0.575	0.416	71	72	72.4	24.6 - 81	24.6 - 98	106	115
XII ▲	504	1070	9.0	335	0.665	0.442	67	68	66.5	21.5 - 85	16.8 - 98	97	106
XII ▲	902	1010	8.2	510	0.565	0.466	79	80	82.4	13.3 - 93	10.4 - 98	98	109
XII ▲	250	1040	7.8	151	0.605	0.406	64	65	67.2	24.5 - 86	21.6 - 98	97	105
XII ▲	545	1010	6.3	350	0.642	0.464	68	71	72.4	23.7 - 94	20.3 - 98	98	107
XII ▲	1030	950	6.7	579	0.562	0.428	83	79	76.2	15.8 - 94	11.6 - 98	105	100

EXPLANATION OF COLUMNS FOR TABLES IIA, IIB AND IIC

REMARKS

Column

1. Experimental group number indicates experiments accomplished using identical sludge. Symbols are those used on Figures 3, 4, 5, 6, 7 and 8.

2. Initial biological solids concentration in mg/l.

3. Initial COD concentration in mg/l.

4. Endogenous respiration, mgO_2/hr/gm initial biological solids.

5. Rate of COD removal during the linear phase calculated as mg/l COD removed/hr.

6. Rate of COD removal during the linear phase calculated as mg/l COD removed/hr/mg/l initial biological solids.

7. Rate of sludge production during the linear phase calculated as mg/l increase in biological solids/hr/mg/l of initial biological solids.

8. Sludge yield in per cent calculated as increase in biological solids divided by COD removed at the time of COD removal, which generally corresponded to the peak in biological solids. When an experiment was terminated before the peak in solids production, the yield was calculated at the last sampling point. The COD removal at this point is shown in column 11.

9. Sludge yield in per cent calculated as increase in biological solids divided by COD removed for each sampling point during the experiment. The average of these individual values is reported.

10. Sludge yield in per cent calculated as rate of biological solids production divided by rate of COD removal during the linear phase; column 7 divided by column 6.

11. Percent theoretical O_2 demand exerted at the indicated percentage of COD removal. Initial COD taken as theoretical O_2 demand.

12. Percent theoretical O_2 demand exerted at the indicated percentage of glucose (anthrone) removal. Initial COD taken as theoretical O_2 demand.

13. & 14. Percent substrate recovery calculated at the end of the experiment using the substrate balance and energy balances previously reported(14). (See text for further explanation.)

the experiments of Group III were run. It is seen that a new relationship between COD removal and initial biological solids was obtained. After this time the color of the unit began to lighten, and at 70 days had nearly attained the golden-brown color it exhibited at 40 days. Experiments in Group IV were conducted, and it is seen that the slope increased but did not return to that found for Group I sludge. Microscopic examination showed that the sludge consisted largely of flocculated individual bacteria, and free-swimming protozoa were again present.

It is emphasized that throughout the experimental period, operational conditions were constant. There were no changes in temperature; pH was maintained at 6.7 ± 0.1, and over 90% dissolved oxygen saturation was maintained at all times.

Unit B

Similar plots of results for experiments conducted on sludge harvested from unit B (1000 mg/l glucose) are shown in Figure 4. The variation in the relationship between COD removal rate and initial biological solids concentration again appeared to be related to changes in predominance. No major changes in the morphological appearance of the systems was noted under the microscope during the 90-day period of operation, but the color of the sludge did undergo changes. Experiments in Groups V, VI, and IX (at 30, 50, and 80 days) were conducted when the unit exhibited a very dark brown color; whereas, when experiments in Groups VII and VIII (at 40 and 60 days) were conducted, the sludge was light or golden-brown.

Unit C

Sludge from unit B was used to seed another batch activated sludge unit which was fed 5000 mg/l glucose (unit C). Three groups of experiments were performed using sludge harvested from this unit. The results are shown in Figure 5. Experiments in Group X were made after 30 days of operation of the unit, while the experiments for Groups XI and XII were performed after 50 and 65 days of operation respectively. As in the previous cases, simple straight line relationships were obtained for identical sludges, but the slope for Group X was considerably different from the slopes for Groups XI and XII. Throughout the period of operation the color of the control

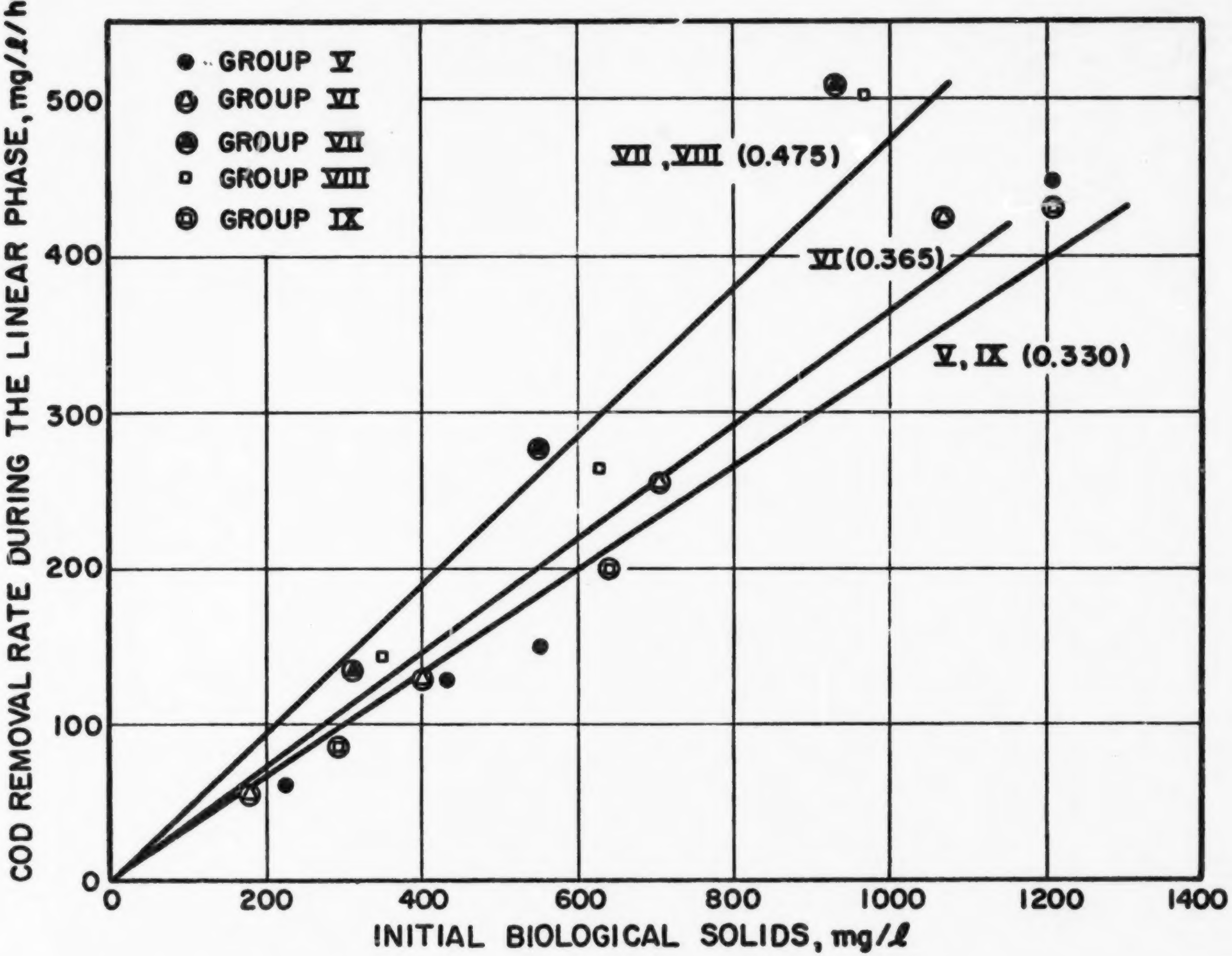

FIG. 4-EFFECT OF INITIAL BIOLOGICAL SOLIDS CONCENTRATION ON THE RATE OF SUBSTRATE REMOVAL; SLUDGE TAKEN FROM UNIT B AT VARIOUS TIMES DURING A 90 DAY PERIOD OF OPERATION.

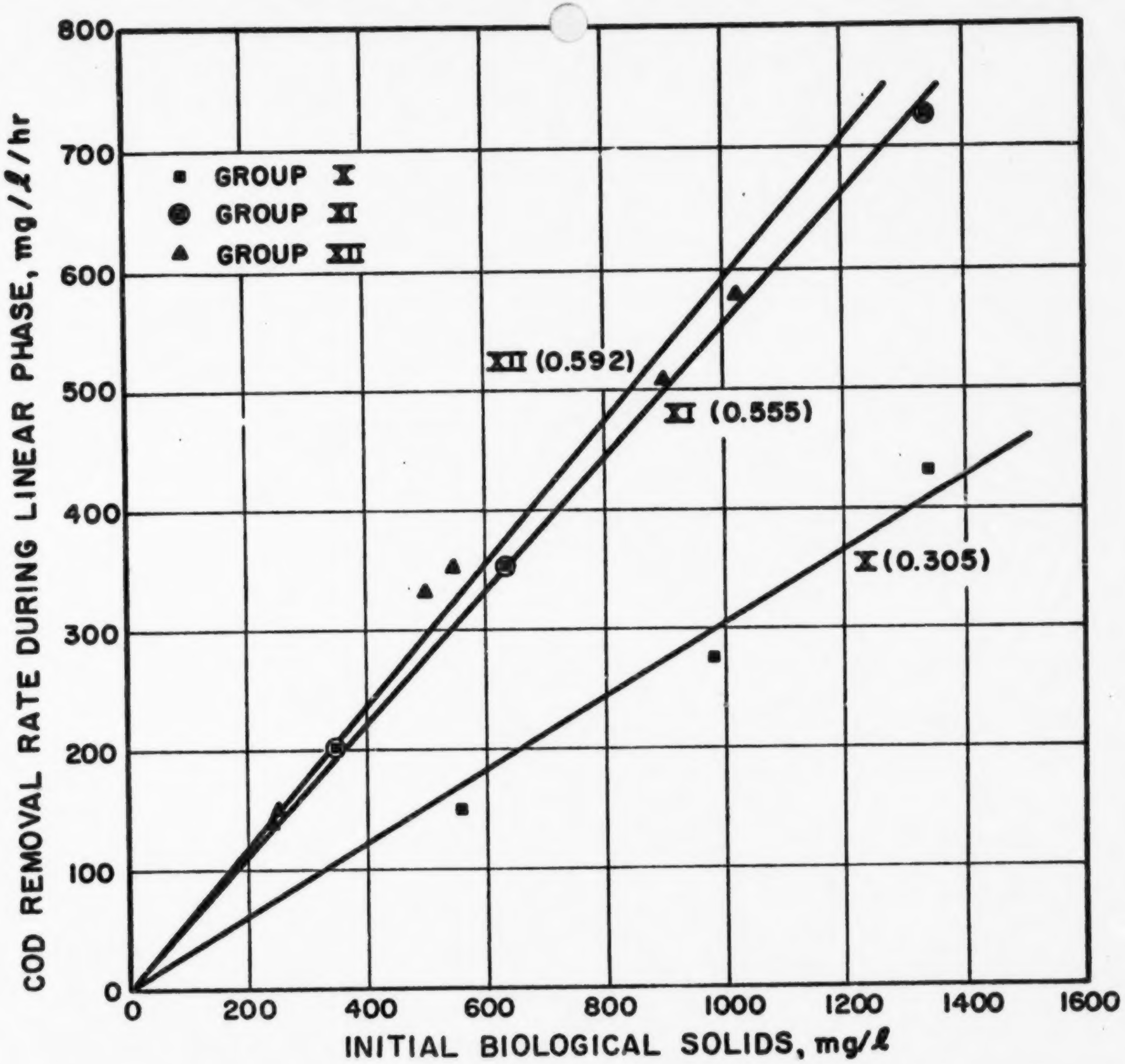

FIG. 5 - EFFECT OF INITIAL BIOLOGICAL SOLIDS CONCENTRATION ON THE RATE OF SUBSTRATE REMOVAL; SLUDGE TAKEN FROM UNIT C AT VARIOUS TIMES DURING A 90 DAY PERIOD OF OPERATION.

unit remained dark brown. There was no severe change in the morphological appearance of the sludge, except that there appeared to be greater numbers of filamentous forms present at the time of the experiments in Group X.

Sludge Yields

In Tables IIA, B, and C the sludge yields calculated by three methods are shown. The method reported in column 8 is the one usually employed; i.e., the yield is calculated at the time of maximum solids production unless the experiment was terminated before the time of COD removal. In calculating the yields reported in columns 9 and 10, the period of active growth and substrate removal was taken into account. Column 9 represents the average of yields calculated at each sampling point; column 10 represents the yields calculated from the slopes of the sludge growth and substrate removal curves. Thus the cell yields reported in column 10 represent the ratio of the sludge growth rate (mg/l cells/hr/mg/l initial cells) and the substrate removal rate (mg/l COD/hr/mg/l initial cells). These yields for all experiments are plotted in Figure 6. The slope of the line of best fit indicates a sludge yield of 0.651; however, it is seen that there is a considerable dispersion of the points even though the operational conditions remained constant and only one substrate (glucose) was used.

Substrate Respiration

Substrate removal was measured by both COD analysis and the anthrone test, and oxygen uptake was measured for each system as well as for an endogenous sludge blank. It was of interest to examine the constancy of the percentage of the theoretical oxygen demand exerted at the time of COD removal and of glucose removal in relation to the initial biological solids concentration.

Figure 7 shows the percentage of the theoretical O_2 demand (initial COD) exerted at the time of COD removal for all experiments except those which were terminated before 94% of the COD had been removed, plotted against initial biological solids concentration. Values of this parameter varied from approximately 12 to 30%, and there was a general downward trend as initial solids concentration increased.

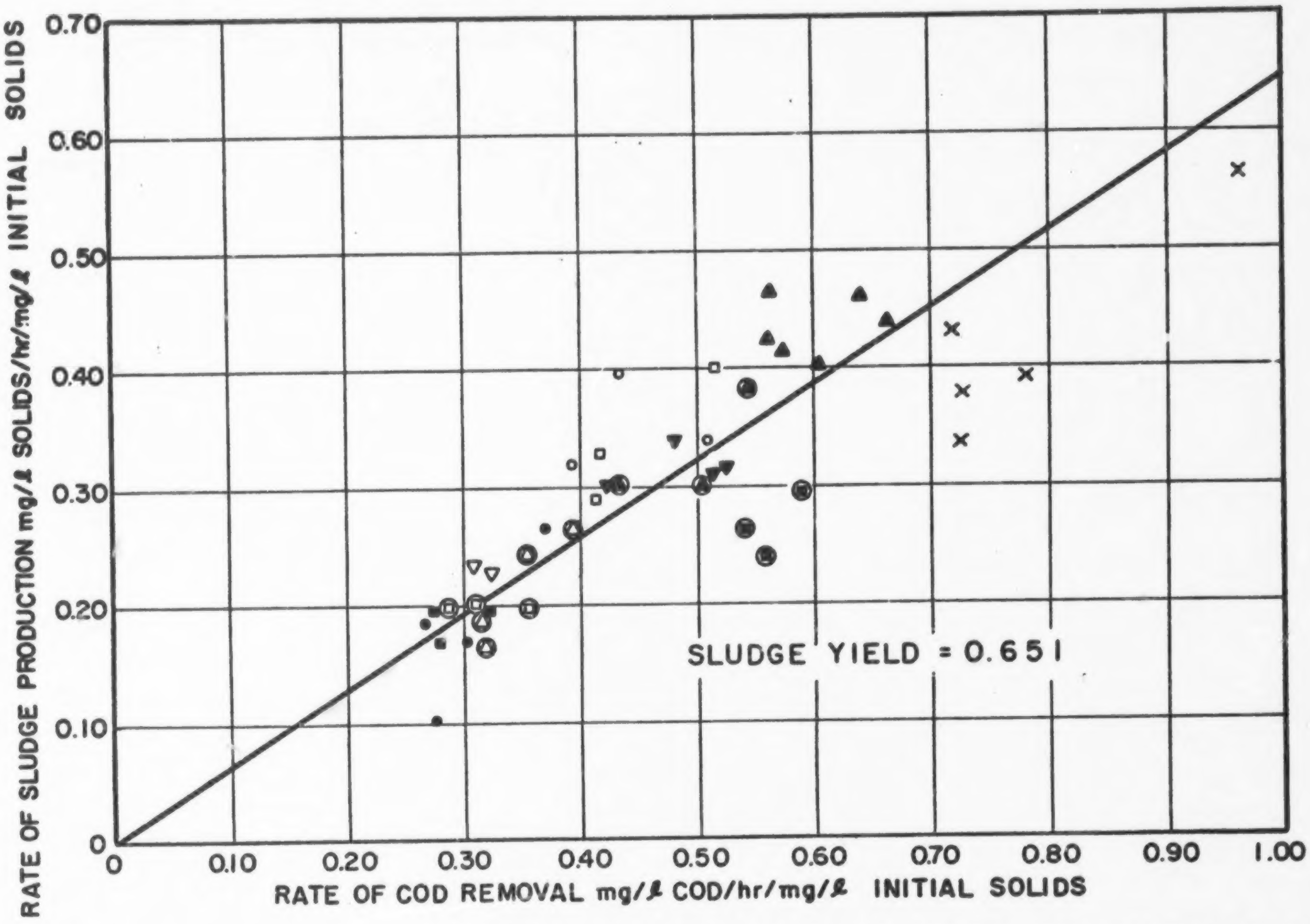

FIG. 6-RELATIONSHIP BETWEEN RATES OF SLUDGE PRODUCTION AND COD REMOVAL (SLUDGE YIELD).

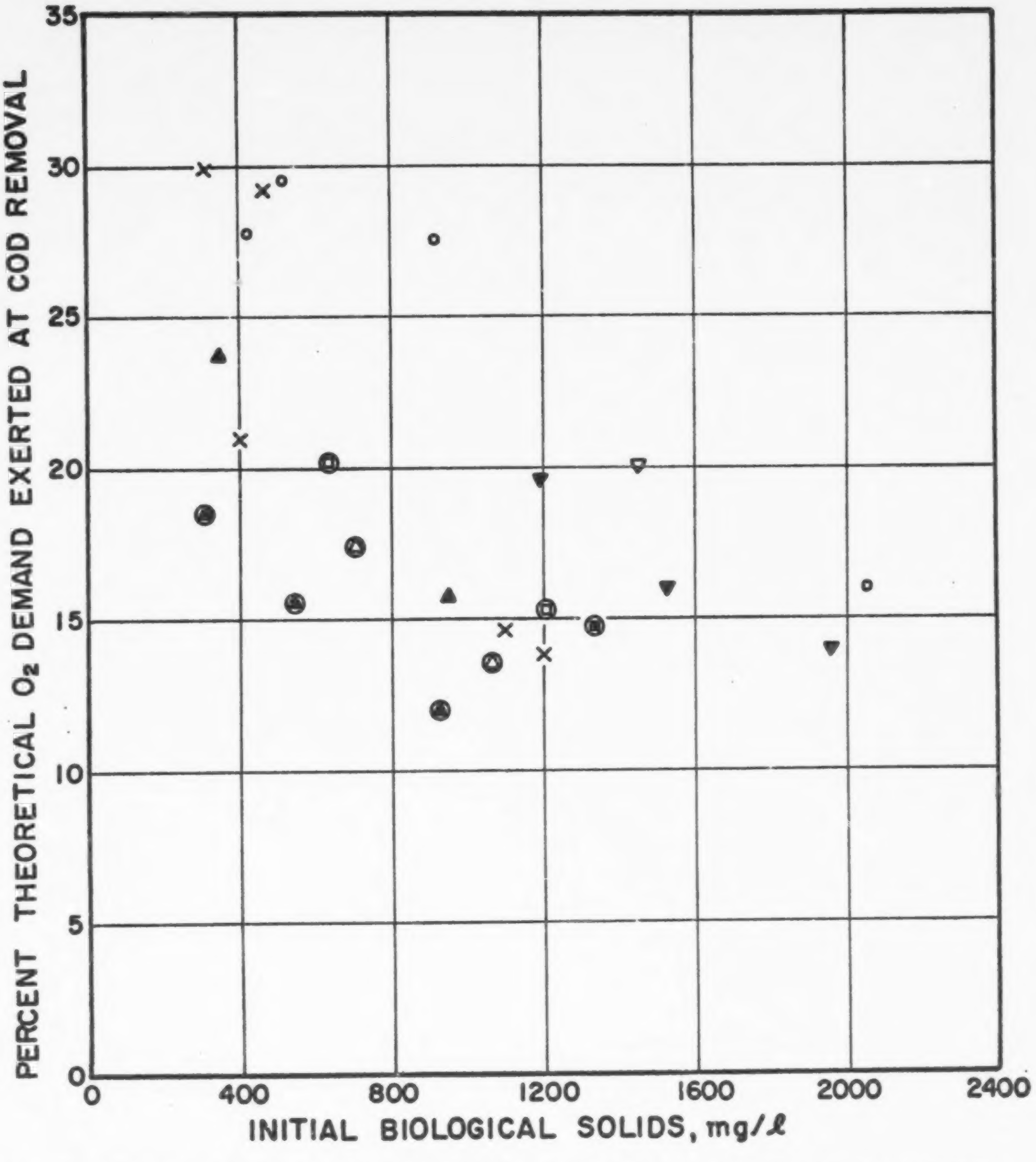

FIG. 7 - EFFECT OF INITIAL BIOLOGICAL SOLIDS ON PERCENTAGE OF THEORETICAL OXYGEN DEMAND EXERTED AT TIME OF COD REMOVAL (94% OR ABOVE TAKEN AS COMPLETE SUBSTRATE REMOVAL)

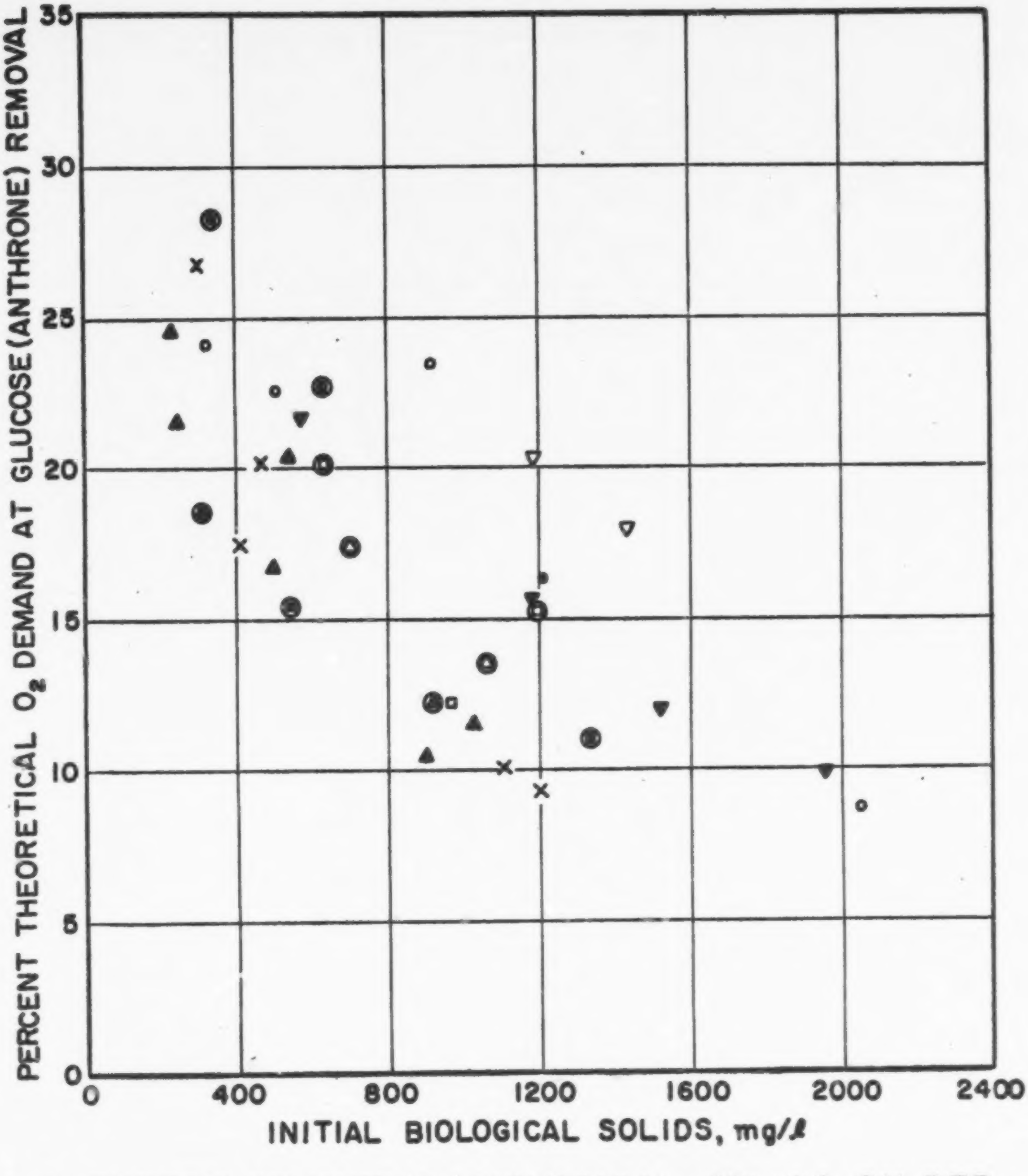

FIG. 8 - EFFECT OF INITIAL BIOLOGICAL SOLIDS ON PERCENTAGE OF THEORETICAL OXYGEN DEMAND EXERTED AT TIME OF GLUCOSE (ANTHRONE) REMOVAL (98% TAKEN AS COMPLETE SUBSTRATE REMOVAL).

Figure 8 is a similar plot in which glucose (anthrone) rather than COD is compared to initial biological solids concentration. The per cent theoretical O_2 demand exerted at the time of glucose removal varied from approximately 8 to 28 per cent. As in the case of COD removal (Figure 7), the lower values were obtained at the higher initial biological solids concentrations. The per cent theoretical oxygen uptake values from which Figures 7 and 8 were plotted are shown in columns 11 and 12 in Tables IIA, B, and C. The endogenous O_2 uptake rate per mg/l initial solids for all experiments is shown in column 4. This parameter for a specific sludge remained fairly constant regardless of the sludge concentration.

Materials and energy balances are given in columns 13 and 14. Figures given in column 13 represent percent substrate recovery on a weight basis using a method previously described(7). Column 14 shows percent recoveries calculated by the energy balance method previously described using a COD to cell weight ratio of 1.2 found experimentally for old cell sludge(14).

DISCUSSION

Initial Solids vs COD

It can be seen from the results of each experiment that when increasing amounts of a single sludge were used as initial seeding material the rate of substrate removal was directly proportional to the initial solids concentration. However, these results demonstrate that the relationship between initial biological solids concentration and substrate removal is not constant for a given substrate or type of waste. It is emphasized that in the present studies only glucose was used as substrate, and the batch activated sludge units were operated under very closely controlled conditions. Therefore, if there is a single straight line relationship for any given substrate or type of waste, it would be expected that it would have been observed in these studies. However, the results shown in Figures 3, 4, and 5 indicate a range of slope between 0.305 and 0.745 for glucose. These figures represent the range for a single carbon source under conditions of prolonged operation, and emphasize the importance of long term laboratory investigations in assigning a quantitative value to such an important design criterion as the relationship of biological solids concentration to rate of waste water purification.

Based upon microscopic observation and changes in the color of the aerating suspension, this rather wide variation in the slope of the relationship is attributed to observed changes in predominance during the experimental period. It is realized that microscopic observation is not a very precise measurement of changes in bacterial predominance, since large changes in predominance can occur without easily observed changes in morphology. However, when there are drastic morphological changes in the microbial forms present in a sample, there can be little doubt that predominance changes have occurred. It might be argued that changes in the color of the aeration liquor may not be indicative of gross changes in predominance; however, when the color change is correlated to gross changes in the microscopic forms present in the sample as observed under the microscope, such changes in color would appear to provide a good indication of changes in predominance. Changes in the color of an aerating suspension have also been used to assess changes in bacterial predominance by other workers(15).

The conclusion that a range of slopes (rather than a constant slope) was due to changes in predominance of microorganisms during the experimental period poses an important and as yet unanswered question. It is well known that changes in the operational or cultural conditions during growth can foster the predominance of species best suited to the particular cultural conditions employed. In the present study the operational and cultural conditions were maintained within the limits of constancy allowed by batch operation. It must be reasoned, then, that the changes in predominance are random and are brought about solely by interaction between the organisms. Some work to determine the reasons for such changes in bacterial predominance under constant operational conditions in both batch and continuous flow systems has been accomplished in the authors' laboratory(16), but this problem warrants an extensive long term research effort.

Cell Yield

Concerning cell yield, it is seen in columns 8, 9, and 10 of Tables IIA, B, and C that there was considerable variation in this important operational parameter, and that this variation was fairly random; i.e., lower yields were not obtained at higher initial cell concentrations. This tends to indicate that if there is a maintenance requirement, i.e., the use of exogenous substrate simply to satisfy the energy and possible resynthesis requirements of intact cells already in the system, it was not manifested at the initial cell concentrations employed in these studies (up to 2000 mg/l). The average cell yields computed by all of the three methods employed (columns 8, 9, and 10) agree fairly well. These averages were 65, 67, and 65 per cent, respectively, for columns 8, 9, and 10. The coefficients of variation for yields computed in accordance with the methods of columns 8 and 9 were approximately 14% for each, and 17% for those of column 10. This indicates a statistical range of yields from 48 to 82% for cells grown on glucose minimal medium using ammonium ion as the source of nitrogen. This range of sludge yields fits the bulk of the yield data reported in the literature, but is considerably higher than the yield obtained by Servizi and Bogan(11) and by McWhorter and Heukelekian(9). Based upon these results, it seems unreasonable to present specific figures for the relation between yield and COD or free energy without also giving the normal range of variation of these figures. For example, based upon the work herein reported, the relationship between cell yield and COD could be expressed as:

$$Y = k(\text{COD})$$

$$\text{where } k = 0.65 \pm 0.17$$

that is, in giving such an equation it is emphasized that $k = 0.65$ is not a constant. The present work shows that much of the variation in yields reported in the literature is not necessarily the result of different experimental conditions, but is most probably the result of random variation in yield due to variations in predominance of the microbial population. It would appear that it is a totally gross simplification to expect that the cell yield is solely a function of the structure of the substrate or the free energy of the substrate. The efficiency of oxidative phosphorylation (the major source of ATP) is not known for most bacteria and ATP yield may be greater or less in different bacteria, depending upon this factor(17). Even if all aerobic organisms obtained energy via the same metabolic pathway and achieved the same P/O ratio in electron transport, thus having the opportunity to obtain the same amount of ATP, it has not been shown that the efficiency of coupling the ATP yield to fruitful biosynthesis is the same for all organisms as has been intimated in the recent literature. Furthermore, according to the Douderoff hypothesis, aerobic growth is limited by availability of carbon, not by available energy(18).

Substrate Respiration

McWhorter and Heukelekian found that the removal of glucose approached completion at 18% of the theoretical oxygen uptake of the substrate, regardless of the initial seed concentration. The results of the present study do not agree with their findings since the percentage of the theoretical O_2 demand exerted at glucose removal is not constant, but decreases with increasing concentration of initial biological solids.

The same trend was also found for COD removal. Concerning this parameter, there is agreement with the work of McWhorter and Heukelekian. In their studies the percentage of the theoretical oxygen demand exerted at COD removal decreased from 30% using a small inoculum (0.5% sewage seed) to 21% using 1010 mg/l activated sludge. In the present study, in which many experiments were run, it was seen (Figure 7) that this parameter varied for different sludges at the same initial solids concentration and that for each specific sludge there was in general a decrease in percent theoretical oxygen uptake exerted as the initial solids concentration was increased. This parameter ranged from a high of approximately 30% at low sludge concentrations to approximately 12% at high concentrations. The percent theoretical O_2 demand exerted at glucose removal ranged from a high of approximately 28% at low cell concentrations to approximately 9% at the higher cell concentrations, and again there was considerable variation in the values for different sludge samples. The fact that the percentage of theoretical oxygen demand expressed at COD removal is higher than at glucose removal would appear to be due to the release of biochemical intermediates or end products.

Based upon the present findings and previous work, it is possible to formulate a fundamental theory which explains why the percentage of the theoretical oxygen demand at both glucose and COD removal decreases with increasing initial solids concentration. Sludge harvested from a batch system at some time after the substrate has been removed, has existed under starvation or endogenous conditions. Such cells are thus depleted of carbon stores or pool substances, and many enzymes needed for balanced replication processes may have been broken down in order to help provide for the endogenous energy requirement of the cells(19). When these cells are contacted with a fresh supply of the same carbon source on which they were originally grown, there is a period during which the cells grow in the sense that they increase in mass but do not yet begin to replicate. This period (the lag period) is usually considered as one of adjustment to the new environment and is a time when required intermediates and pool substances build up to the point where synthesis of materials needed for replication can begin(19)(20). During this time much substrate can be assimilated, depending upon the initial inoculum of cells present in the system. Indeed, substrate must be metabolized in order to replenish metabolic pools of building blocks the organisms need to synthesize protein, nucleic acids, and structural carbohydrate and lipid before exponential replication can proceed. It seems apparent that the larger the initial inoculum, the greater will be the percentage of total substrate assimilated in this preparatory stage. In this phase it might be expected that the primary synthesis product would be carbohydrate, since these materials are less complicated and require fewer conversion steps, especially when the substrate itself is a carbohydrate. These carbohydrate synthesis products also serve as carbon source for the synthesis of protein, nucleic acid, etc. Thus synthesis of a non-nitrogenous nature, e.g., carbohydrate, would precede synthesis of nitrogenous material needed for replication. There are published results which show that protein synthesis, although occurring concurrently with carbohydrate synthesis, does, on a proportional basis, lag carbohydrate production. Gaudy and Engelbrecht have presented data on cell composition during glucose metabolism in batch systems under growth conditions(7)(21). Using these results, the protein and carbohydrate content of the sludge can be computed for the various sampling points. These values are shown in Table III. It is seen that there was a proportionally greater synthesis of carbohydrate than protein during the early hours of the study, and during this time most (or all) of the carbon source was removed. Komolrit and Gaudy have found that this same effect was evidenced during sorbitol metabolism(22).

It is also interesting to compare the percentage of the theoretical oxygen demand exerted at the point of substrate removal for growing and nonproliferating systems. In a nonproliferating system nitrogen is not added; thus there is no opportunity for a net production of compounds, e.g.,, protein, which have a high energy of synthesis requirement. Thus it might be expected that the percentage of the theoretical O_2 demand exerted at substrate removal would be lower than that for a growth system. Again, such a comparison is provided through analysis of results previously presented by Gaudy and Engelbrecht(7)(21). Both nonproliferating and growing systems were run using equal portions of an identical sludge. The increase in sludge weight in both systems was approximately the same and it was shown that this increase in the nonproliferating system was due primarily to synthesis of carbohydrate. While a slightly longer time was required to remove the carbon source in the nonproliferating system, the percent of the theoretical O_2 demand exerted at COD removal in this system was 18.3 per cent, while for the growing system it was 23.3 per cent.

These results are consistent with the fact that the synthesis of a carbohydrate store requires considerably less energy than does production of protein and nucleic acid. Also, before the cells can synthesize these more complicated compounds, they must first produce the enzymes required for their production. Hence, in a growing system the early synthesis of low energy-requiring compounds is a result which might be anticipated from a basic biochemical and thermodynamic standpoint. It follows that the synthesis of such compounds would require less oxygen, since oxygen is the primary final electron acceptor in aerobic systems. These processes occur in all growing systems, regardless of the size of the initial inoculum;

TABLE III

PERCENT CARBOHYDRATE AND PROTEIN CONTENT OF SLUDGE DURING METABOLISM OF GLUCOSE BY BATCH ACTIVATED SLUDGE

Analysis	Time, hours							
	0	1.5	3.0	4.5	6.0	12	23	36
COD remaining mg/l	506	199	45	35	43	45	45	55
Sludge * Protein %	45	41	40	43	57	48	45.6	44.5
Sludge * Carbohydrate %	18.3	23	24	21.5	15	16.8	17.5	17.3

*Calculated from protein, carbohydrate and biological solids data presented in Figure 5a, Gaudy and Engelbrecht(21) or Figure 3, Gaudy and Engelbrecht(7). Calculations were based upon the recorded values from which these figures were plotted(23).

however, when the initial cell inoculum is low, measurable oxygen uptake cannot be registered without considerable replication of the cells, i.e., balanced synthesis of all cellular constituents leading to a proportionally higher energy requirement. As the initial level of biological solids increases, so does the number of cells participating in low energy-requiring synthesis (e.g., carbohydrate). Thus more of the carbon source is removed for this type of synthesis, and the percentage of the theoretical oxygen demand of the substrate exerted at substrate removal must therefore decrease as initial biological solids increases. This hypothesis is substantiated by: (1) the results of the present study; (2) the known response of somewhat starved cells when placed in fresh medium; and (3) demonstration of proportionally greater synthesis of carbohydrate than protein as cited above.

This hypothesis has fundamental ramifications toward understanding the biosorption process wherein essentially starved cells are contacted for a short time with fresh substrate, and it tends to explain why tremendous increases in the air requirement may not be observed in the contact tank.

CONCLUSIONS

Extensive, long-term studies have shown that there is a straight-line relationship between initial solids concentration and substrate removal rate for a specific sludge. However, for sludge samples taken at different times from a unit operated over a long period, the following observations were made:

1) The relationship between initial solids concentration and rate of COD removal varies for a single substrate.

2) The cell yield varies for a single substrate.

3) The percentage of theoretical oxygen demand exerted at the point of both glucose and COD removal decreases with increasing initial solids concentrations.

The variations in relationship between solids and purification rate and in cell yield appear to be due to changes in predominance in the population which occur in spite of controlled, constant operational conditions. The decrease in percent theoretical oxygen demand exerted at substrate removal when initial solids are increased can be explained on the basis of differences in the amount of the total substrate utilized in early synthesis of carbohydrates. This synthesis, which occurs when starved cells are placed in fresh medium, requires less energy and therefore consumes less oxygen than do synthetic processes which take place after the cells have become adjusted to the new environment.

ACKNOWLEDGEMENT

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PAPER 14

REGULATION OF EXOGENOUS NITROGEN SUPPLY AND ITS POSSIBLE APPLICATIONS TO THE ACTIVATED SLUDGE PROCESS

By

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REGULATION OF EXOGENOUS NITROGEN SUPPLY AND ITS POSSIBLE APPLICATIONS TO THE ACTIVATED SLUDGE PROCESS

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The need for addition of a usable source of nitrogen in the biological treatment of many industrial wastes is well known, and it is general practice to maintain the nitrogen supply at a BOD:nitrogen ratio of 20:1 by addition of nitrogen to the waste. For wastes totally deficient in nitrogen, it has been stated that the cost for this supplementation may be comparable to the power cost for the compressed air when the activated sludge process is employed(1). Often portions of the nitrogen are found in the effluent as nitrite and nitrate, which can enhance excessive algal growth in the receiving stream, thereby negating some of the beneficial effects of

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Paper presented at the 38th Annual Conference, Water Pollution Control Federation, Atlantic City, New Jersey, October 10-14, 1965.

biological treatment. It was felt that it might be feasible to reduce the nitrogen supply by controlled addition to the concentrated sludge rather than continual addition to the incoming waste. The feasibility of such a mode of operation would be based upon the ability of the sludge to assimilate waste constituents for periods of time in the absence of an exogenous nitrogen source (nonproliferating conditions).

Work previously reported lends some credence to this possibility. Using a synthetic carbohydrate waste in which glucose was the carbon source, Gaudy and Engelbrecht(2) showed that the substrate removal achieved by an activated sludge devoid of an extracellular source of nitrogen was comparable to substrate removal by a system in which the nitrogen source was added in excess (growing conditions). However, they warned that a nitrogen deficient system could not be expected to give comparable substrate removal for prolonged periods without the addition of a nitrogen source. In these studies the major difference in the biochemical composition of the two sludges was that under nonproliferating conditions carbohydrate was the major constituent synthesized, whereas under growing conditions protein was the major synthesis product. These findings have recently been confirmed by van Gils(3). Rama Rao, Speece, and Engelbrecht(1) also obtained comparable substrate removal efficiency for nitrogen-deficient and nitrogen-supplemented systems using such substrates as acetate and phenol, as well as glucose. In addition, comparable sludge settleability was observed. They proposed an activated sludge flow sheet for treatment of nitrogen-deficient wastes in which the effluent from the primary settling tank was divided into two parts and channelled to activated sludge plants operated in parallel. One waste stream was supplemented with nitrogen and treated in the conventional manner. One portion of the sludge from the settling tank of this unit was then used as return sludge for the aerator, and the remaining sludge was mixed with a portion of the waste to which no nitrogen was added and thence channelled to the second activated sludge plant. All sludge from the settling tank of the second plant was wasted for final disposal. While this system appeared to have merit and effected a savings in nitrogen, it required a duplicate activated sludge plant.

In the present work a different approach was taken. It was reasoned that an activated sludge system might be devised to take greater advantage of the cells' ability to remove substrate under nonproliferating conditions. For example, if an activated sludge were to be brought in contact with waste devoid of an exogenous nitrogen source, and if the waste could be assimilated under this nonproliferating condition and stored as carbohydrate, it might be possible, after settling the sludge, to convert the storage products to protein in an endogenous phase by adding a nitrogen source to the concentrated sludge, thus in effect regenerating it to a protein-rich sludge. If so, such a sludge (or a portion of it) might then be recycled for another pass with incoming waste deficient in nitrogen. A flow sheet for such a system is shown in Figure 1. It is seen that the purified waste leaves the system without ever coming into contact with the exogenous nitrogen supply, thus helping to prevent the leakage of nitrogen to the receiving stream. In addition, some savings in the nitrogen cost should be effected, since nitrogen is not added to the excess sludge. A system such as this might be applied over the entire range of activated sludge modifications. The possibilities for operating an activated sludge plant in accordance with the flow sheet shown in Figure 1 are based upon the assumption that the carbon which is channelled to synthesis of either internal or external carbonaceous storage products under nonproliferating conditions during the period of substrate removal can be readily converted to protein for cell replication under endogenous conditions in which only a nitrogen source is added. From previous work(4) there was some indication that such interconversion under the above conditions might be possible. Using sludge which had been developed in a continuous flow unit on glucose under conditions of low nitrogen supplementation, it was found that O_2 uptake during endogenous metabolism in the presence of an ample supply of nitrogen was significantly greater than that under endogenous conditions in the absence of exogenous nitrogen. Based upon these results it was felt that an endogenous store built up in a nitrogen-poor environment could be used for synthesis of protein under conditions of plenteous nitrogen source. If this were true, there would be some indication that the proposed mode of operation

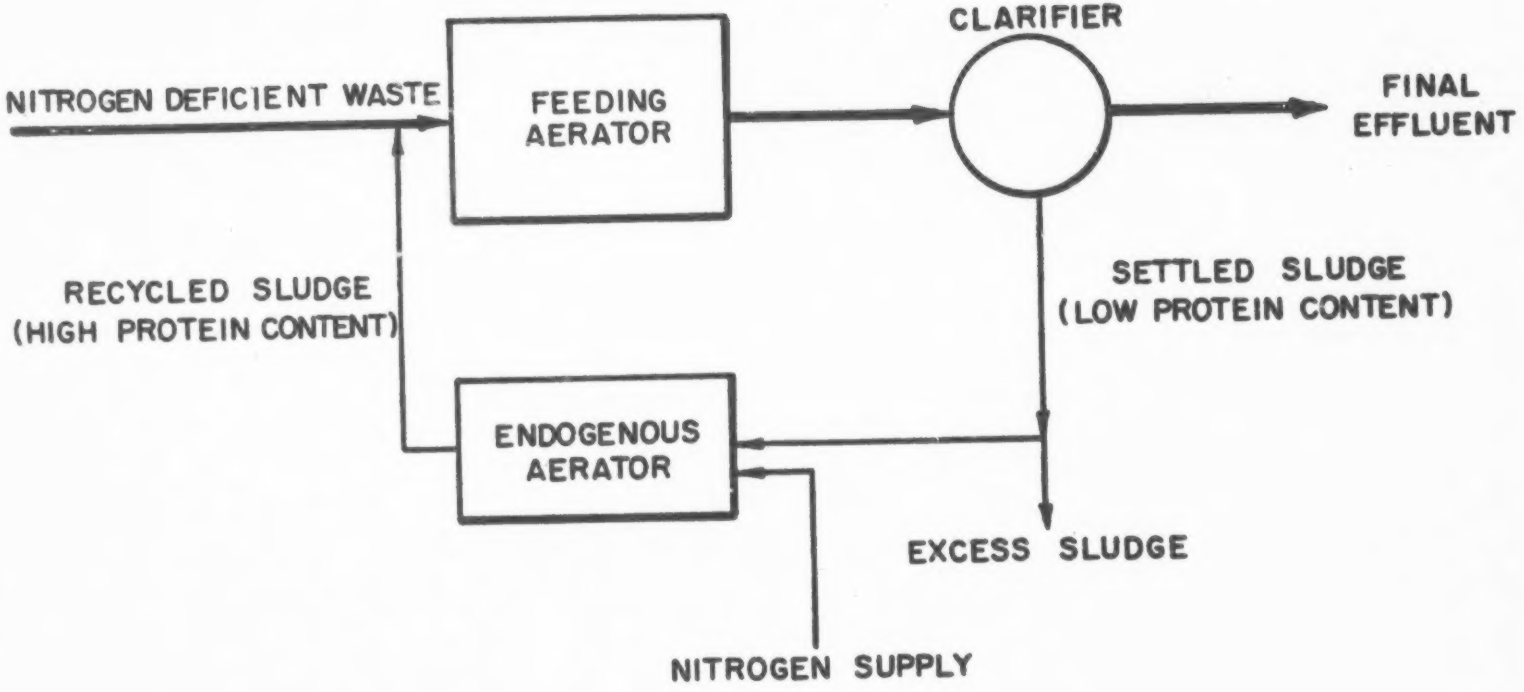
CLARIFIER
NITROGEN DEFICIENT WASTE
FEEDING AERATOR
FINAL EFFLUENT
RECYCLED SLUDGE (HIGH PROTEIN CONTENT)
SETTLED SLUDGE (LOW PROTEIN CONTENT)
ENDOGENOUS AERATOR
EXCESS SLUDGE
NITROGEN SUPPLY

Figure 1 - PROPOSED ACTIVATED SLUDGE PROCESS FOR TREATMENT OF NITROGEN DEFICIENT WASTE.

might be feasible for some types of wastes, and the work herein reported is primarily concerned with the examination of this basic surmise.

MATERIALS AND METHODS

Experimental Design

The activated sludge used in these studies was developed in a laboratory batch unit started from a sewage seed in accordance with the following procedure: Daily, one-third (500 ml) of the total mixed liquor were wasted, the remainder was settled for one hour, and 500 ml of supernatant were discarded. The volume of aeration liquor was made up to the original 1.5-liter mark with synthetic waste composed of the following constituents: glucose, 2000 mg/l; 1.0 M potassium phosphate buffer, pH 7.0, 20 ml/l; $(NH_4)_2SO_4$, 1000 mg/l; $MgSO_4.7\ H_2O$, 200 mg/l; $FeCl_3.6\ H_2O$, 1.0 mg/l; $MnSO_4.H_2O$, 20 mg/l; $CaCl_2$, 15 mg/l; tap water, 200 ml/l, and distilled water to volume. The activated sludge systems were run at $25^oC. \pm 1^o$ with an air flow rate of 4000 ml/min. After one month of such operation, during which time the unit came into solids balance, the sludge was harvested, washed twice in the above buffer-salts medium devoid of substrate and nitrogen source, and used in batch experiments designed to test the feasibility of the flow diagram shown in Figure 1. These experiments were carried out as described below:

Feeding Phase - The harvested sludge first underwent a feeding phase. In this phase, portions of the sludge were used to set up two batch units; one under growing conditions and one under nonproliferating conditions.

Endogenous Phase - After examining the performance of these systems during the period of substrate removal, the sludge from both the growing and nonproliferating units was harvested and washed, and portions from each were used to examine the biochemical changes occurring under two types of endogenous conditions, i.e., endogenous metabolism in the presence and absence of nitrogen.

Refeeding Phase - After the endogenous phase, the sludges were harvested and then re-fed with medium containing the carbon source but no nitrogen (nonproliferating conditions), in order to compare the degree of restoration of sludge purification efficiency due to addition of nitrogen in the endogenous stage. Thus the feeding, endogenous, and re-feeding phases of the experimental design simulated the flow path of the sludge shown in Figure 1.

Another study was made to determine the regeneration capacity of a sludge after prolonged feeding of glucose in the absence of nitrogen source. In this study no sludge was wasted and the system was made up to volume daily with synthetic waste to replace the aeration liquor removed for sampling on the previous day. With this exception, the experimental protocol was the same as that described above.

Analytical Techniques

During each phase of the study, samples were periodically withdrawn from each system for analysis. Sludge concentrations were determined by the membrane filter technique using a 0.45 μ filter. The sludge was also analyzed for its protein and carbohydrate content, using the biuret and anthrone tests according to the procedures previously reported(5)(6). In some studies where only small samples were available, the Folin-Ciocalteau test was also employed for protein determination of the sludges, since this method requires a smaller sample size than the biuret method. The Folin-Ciocalteau method used in these studies was essentially the same as that given by Clark(7); however, a slight modification was made in the present studies in that the biological sludge, after filtration and washing, was resuspended in a known amount of distilled water and homogenized in a sonic oscillator. An aliquot was then taken for protein analysis. Filtrate from the biological solids determination was used for the measurement of glucose remaining in the medium by the anthrone method(5). Total organic material remaining in the filtrate was measured by the chemical oxygen demand (COD) test according to Standard Methods(8). The respiratory activity of the system was also followed by the use of the Warburg apparatus, which was maintained at 25°C. and at a shaker rate of 100 oscillations/min. Previous experiments have shown the equivalence of experimental conditions in the batch activated sludge units and the Warburg apparatus. At a temperature of 25°C. with an air flow rate of 4000 ml/min in the growth tube and a Warburg shaker rate of 100 oscillations/min, rates of COD removal and solids production were the same in both systems(9).

RESULTS

Experiments to Determine Feasibility of Proposed Flow Sheet

Feeding Phase - Figure 2 shows various characteristics of the growing and the nonproliferating systems under identical operating conditions. It is seen that the COD removal efficiency of both systems was quite comparable up to approximately two hours, after which the removal rate of the nonproliferating system was noticeably retarded. It is also seen that in the growing system the biological solids continued to increase rapidly at an approximately constant rate until the exhaustion of COD, whereas in the respiring system the rate of increase in biological solids was noticeably reduced after approximately two hours. It can also be noted that in both systems, as time elapsed, the difference between glucose and total COD in the filtrate continued to increase, from which it is evident that some metabolic intermediates or end products were liberated during glucose metabolism. In the growing system the metabolic intermediates could be readily metabolized and were reduced to a very low level after about four hours, whereas in the respiring system these intermediates were removed at a slower rate. It is seen that in both systems there was a rapid increase in sludge carbohydrate during substrate removal. However, the carbohydrate content of the sludge in the growing system began to decrease after approximately four hours or at the

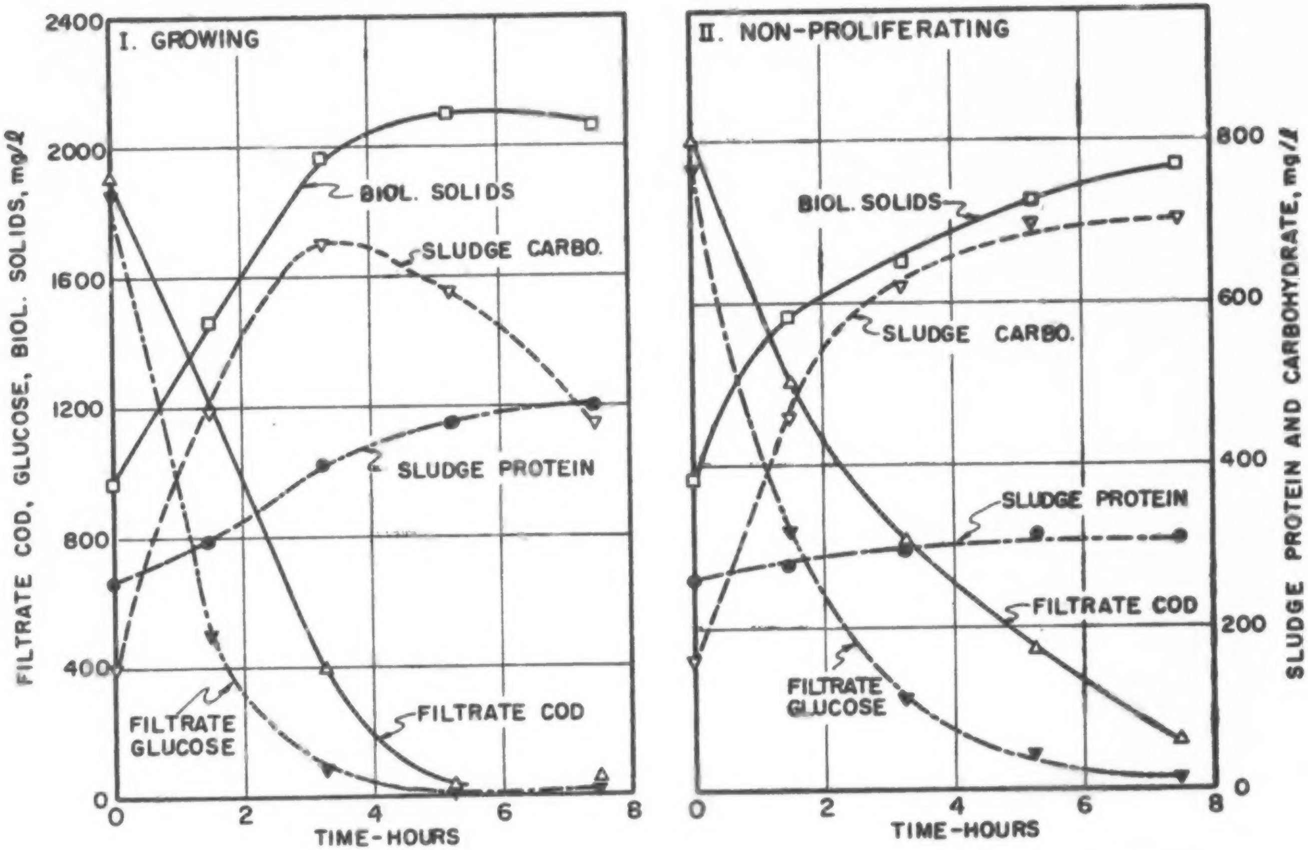

Figure 2 - COMPARISON OF SYSTEM CHARACTERISTICS IN THE FEEDING PHASE UNDER GROWING (I) AND NON-PROLIFERATING CONDITIONS (II).

time of exhaustion of substrate, whereas the protein content continued to increase even after the biological solids had reached its maximum level. The carbohydrate content of the nonproliferating system continued to increase at a rate comparable to that of the increase in biological solids throughout the experimental period. As would be expected, the protein content of the nonproliferating system remained at a relatively constant level. In general, the results are similar to those previously reported by Gaudy and Engelbrecht(2). Respiration of both systems is shown in Figure 3 together with oxygen uptake in the endogenous flasks to which no substrate was added. It is seen that the oxygen uptake of the nonproliferating system was only slightly lower than that of the growth system.

Comparison of the changes in protein and carbohydrate content of the sludge in the growing and nonproliferating systems computed as per cent of biological solids is given in Figure 4. It is obvious that in both cases the systems responded by rapid increase in carbohydrate content in the initial stage, and as a result the protein content of both systems was decreased. After approximately two hours the per cent protein in the growing system began to rise; the carbohydrate content reached a peak, and thence began to decrease. It should be noted that while, by the end of the experiment, approximately equal amounts of substrate had been removed in both systems with approximately equal amounts of biological sludge produced, the protein content

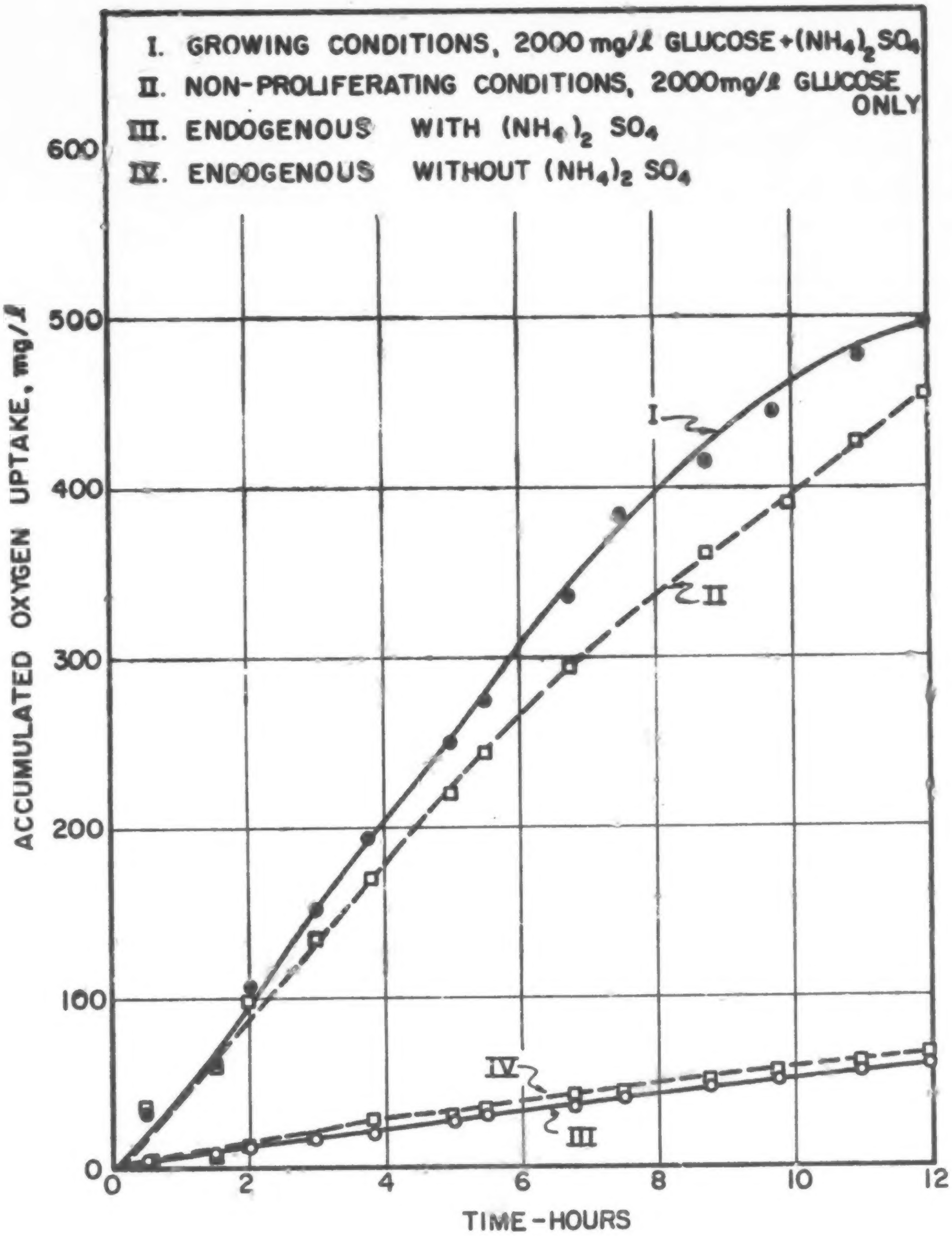

Figure 3-COMPARISON OF OXYGEN UPTAKE IN THE FEEDING PHASE UNDER GROWING AND NON-PROLIFERATING CONDITIONS.

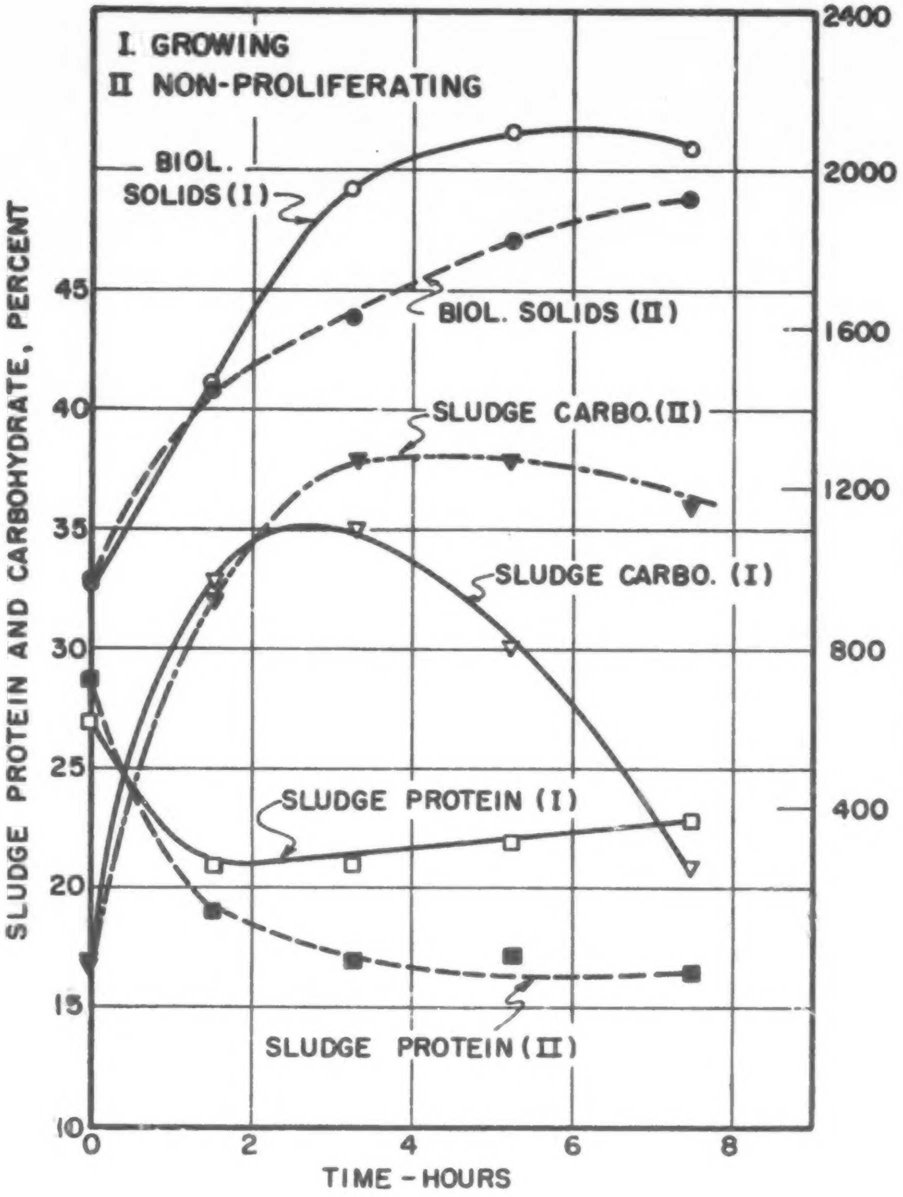

Figure 4 - COMPARISON OF CHANGES IN SLUDGE PROTEIN AND CARBOHYDRATE CONTENT IN THE FEEDING PHASE UNDER GROWING AND NON-PROLIFERATING CONDITIONS.

of the growing system was nearly 1½ times that of the nonproliferating system, and its carbohydrate content was approximately one-half.

Endogenous Phase - At the end of the feeding phase the sludge from both systems was placed under endogenous conditions in the presence and absence of nitrogen source. The results are shown in Figure 5. It is seen that the addition of a nitrogen source to the sludge obtained from the growing system had no significant effect on changes in sludge concentration or in protein and carbohydrate content of the sludge. However, for the sludge harvested from the nonproliferating system, the addition of ammonium ion under endogenous conditions caused a significant decrease in carbohydrate content coupled with an increase in the protein content. This was especially noticeable during the first four hours of the experiment. However, for the same sludge under endogenous conditions without nitrogen source, such a turnover in sludge composition was not apparent.

The respiration of all four systems is shown in Figure 6. It is also evident from this figure that the sludge obtained from the nonproliferating system underwent an extremely active endogenous metabolism in the presence of nitrogen source (see II-A). Indeed, more oxygen uptake was registered in this system than in any other shown.

Refeeding Phase - The activity of the sludges from all four systems was next compared by measuring substrate removal ability in the absence of nitrogen source. The results

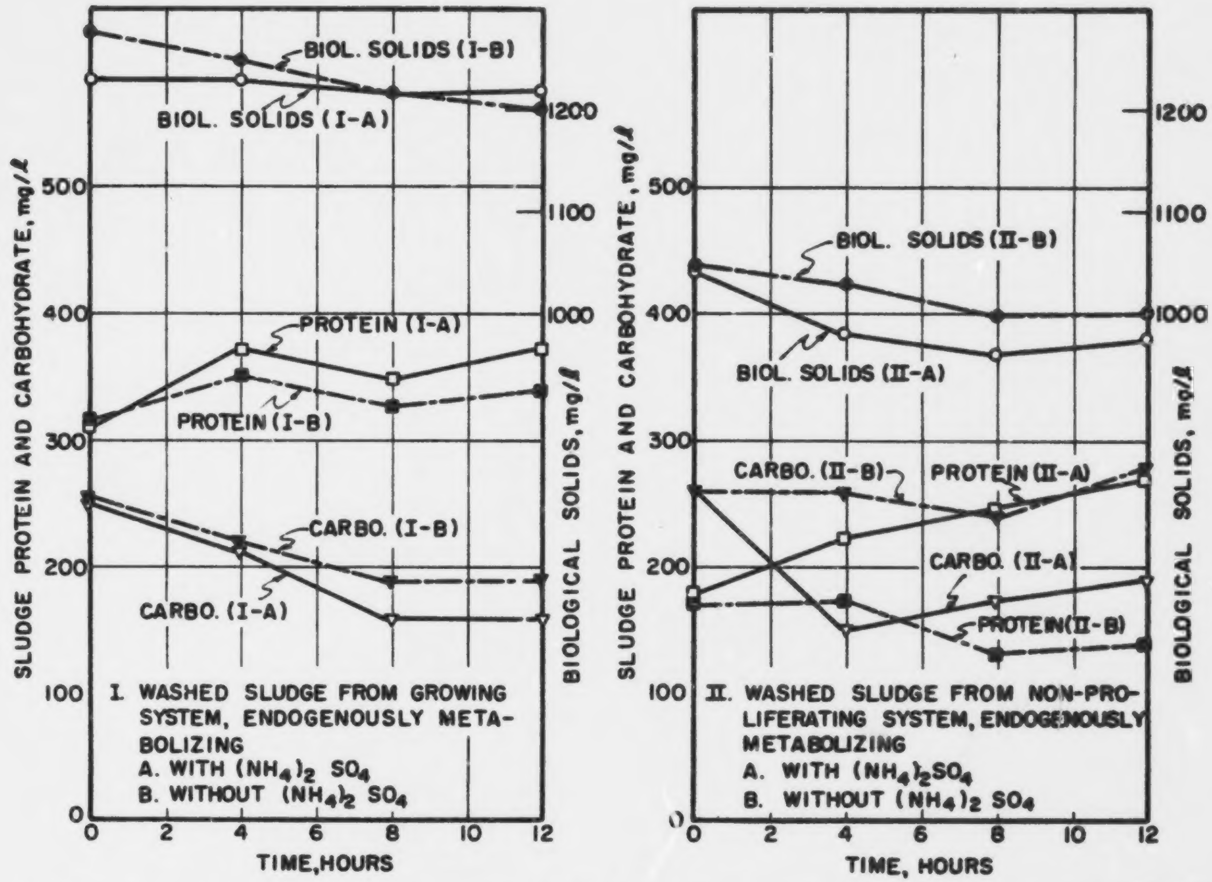

Figure 5 - COMPARISON OF THE CHANGES IN SLUDGE PROTEIN AND CARBOHYDRATE IN THE ENDOGENOUS PHASE WITH AND WITHOUT AMMONIUM SULPHATE.

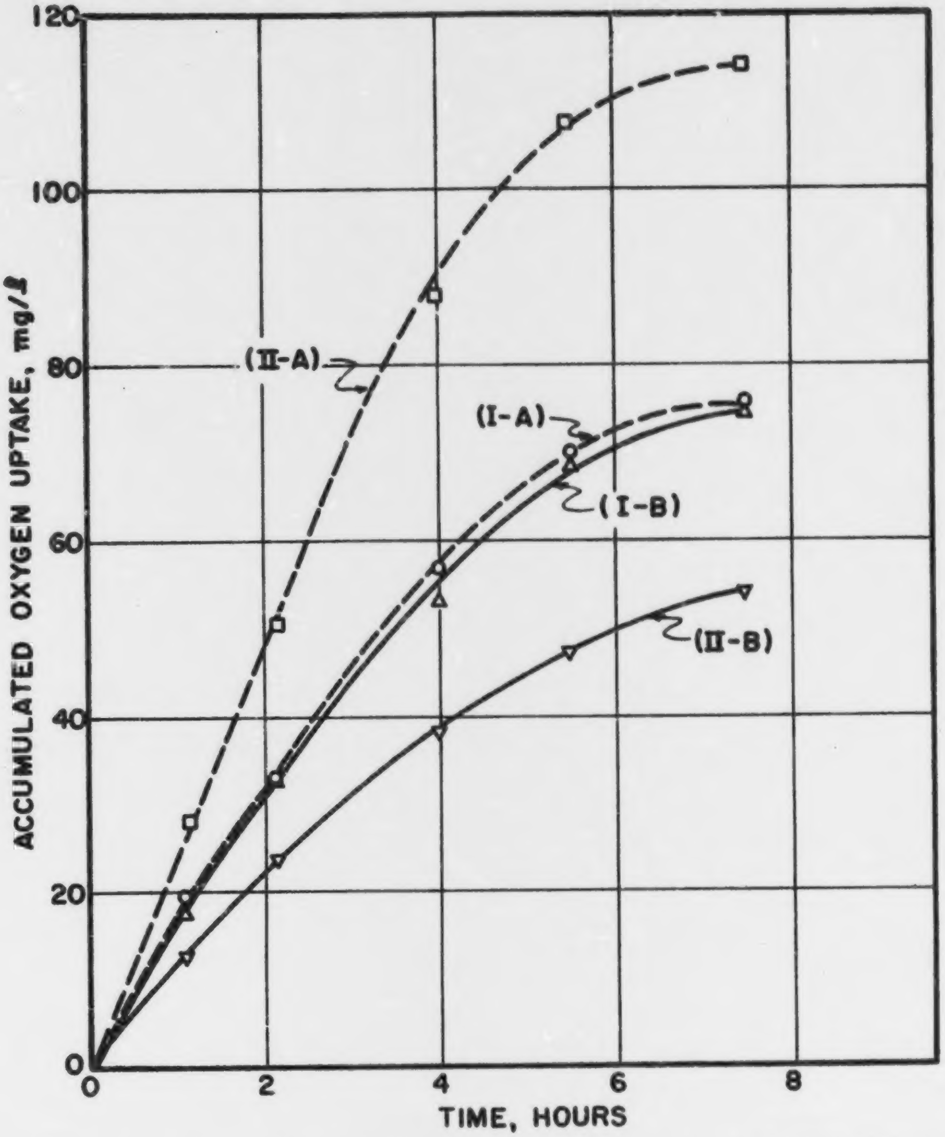

Figure 6 - COMPARISON OF ENDOGENOUS OXYGEN UPTAKE FOR SLUDGE HARVESTED FROM GROWING AND NON-PROLIFERATING SYSTEMS IN THE PRESENCE AND ABSENCE OF AMMONIUM SULPHATE. I-A GROWING SYSTEM SLUDGE WITH $(NH_4)_2SO_4$; I-B GROWING SYSTEM SLUDGE WITH NO $(NH_4)_2SO_4$; II-A NON-PROLIFERATING SYSTEM SLUDGE WITH $(NH_4)_2SO_4$; II-B NON-PROLIFERATING SYSTEM SLUDGE WITH NO $(NH_4)_2SO_4$.

are shown in Figure 7. It is clearly seen that there was no difference in activity of the sludges which were originally harvested from the growing system after being allowed to metabolize endogenously with and without nitrogen source (see Figure 7-I). However, there were significant differences between the activities of the sludges harvested from the nonproliferating system (see Figure 7-II). It is also seen that the activity of sludge(system II-A) which was supplied with ammonium sulfate in the endogenous phase, was very similar to the activity of the sludge harvested from the growing system. The removal efficiency of the system for which ammonium sulfate was omitted in the endogenous phase (II-B) was inferior to all others. This is also borne out by the O_2 uptake curves for these systems, which are shown in Figure 8. The respiration rate of the sludge from the nonproliferating system, exposed to a source of nitrogen only in the endogenous phase, is comparable to the O_2 uptake of the sludge developed under normal growing conditions (compare I-A and I-B with II-A).

The above results provide definite evidence that the material synthesized during substrate removal in the absence of nitrogen underwent conversion to protein during an endogenous phase in the presence of nitrogen, and that this conversion allowed the sludge to regain its capacity to remove substrate under nonproliferating conditions, thus substantiating the feasibility of the mode of operation depicted in Figure 1. However, it seemed evident that a

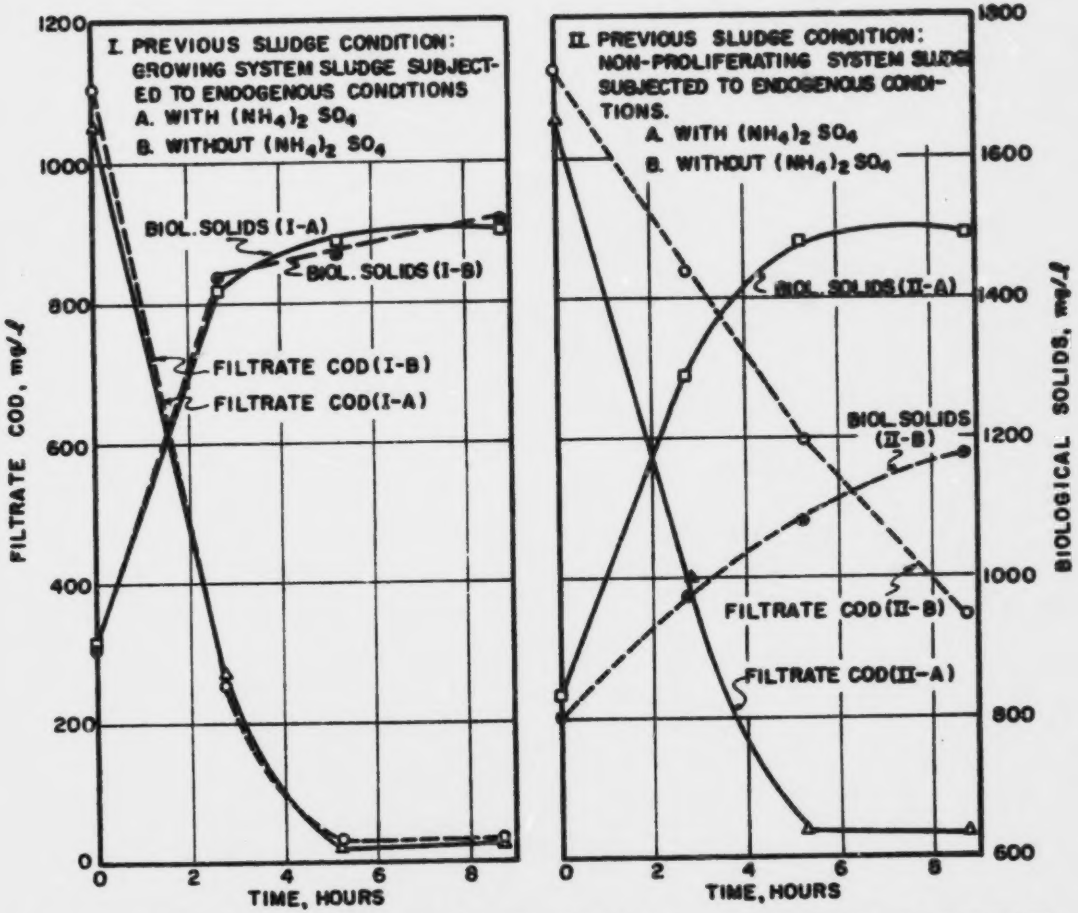

Figure 7 - COMPARISON OF SUBSTRATE REMOVAL UNDER NON-PROLIFERATING CONDITIONS IN THE REFEEDING PHASE AFTER BEING SUBJECTED TO THE PREVIOUS CONDITIONS INDICATED.

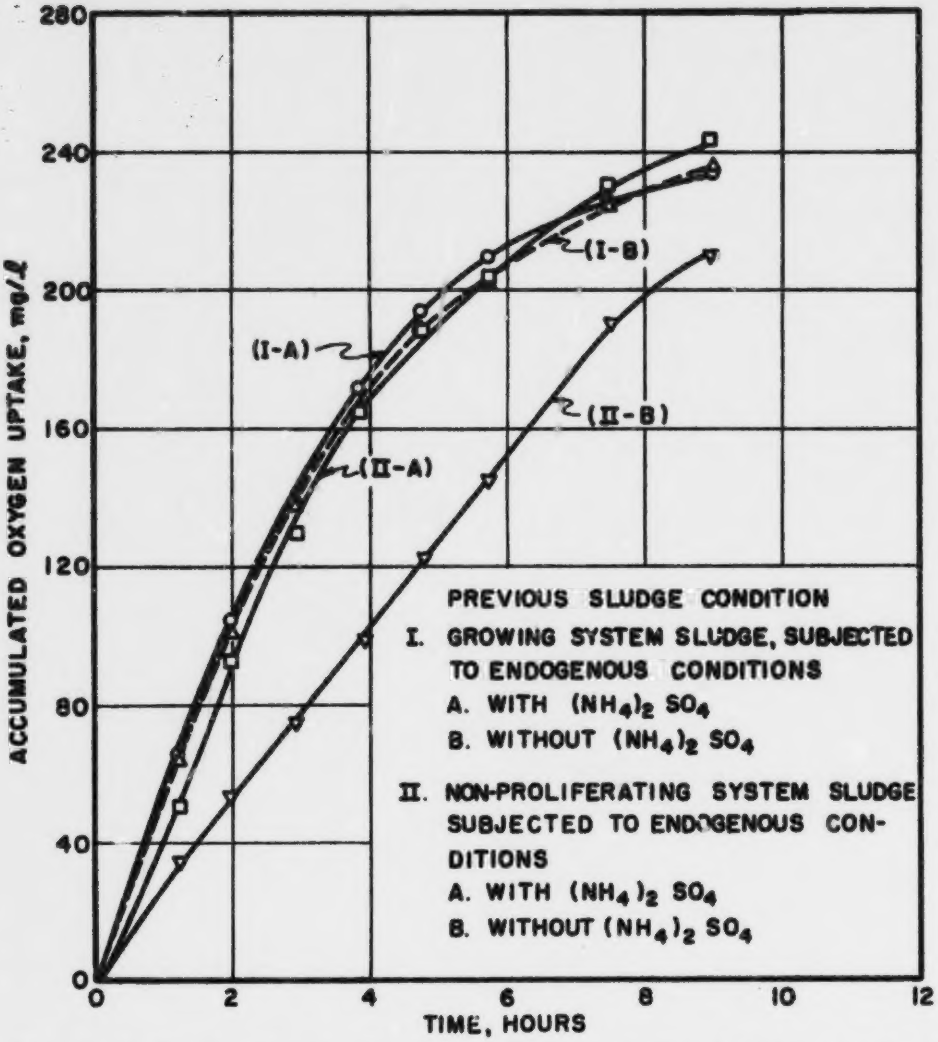

Fig. 8- COMPARISON OF O_2 UPTAKE UNDER NON-PROLIFERATING CONDITIONS IN THE REFEEDING PHASE AFTER BEING SUBJECTED TO THE PREVIOUS CONDITIONS INDICATED.

sludge purposely held devoid of exogenous nitrogen source would lose its substrate-removing capability if not regenerated by addition of nitrogen in the endogenous phase. It was therefore of basic interest to determine the course of deterioration of substrate removal efficiency for prolonged periods of omission of nitrogen, and whether after such deterioration the activity of the sludge could again be regenerated by adding a nitrogen source.

Regeneration of Sludge after Prolonged Feeding in the Absence of a Nitrogen Source

Feeding Phase - Comparison of substrate removal capability during four successive days of feeding sludge under nonproliferating conditions is shown in Figure 9. It is seen that during the first day (comparable to the previous experiments) substrate was readily removed. However, on days 2, 3, and 4, successive deterioration of COD removal efficiency occurred. While glucose was always totally removed at a relatively rapid rate, COD removal became increasingly inefficient and was governed by two rates. The second rate was slower, and was initiated at the point of glucose removal (as measured by anthrone). While the amount of daily sludge accumulation was reduced from that of the first day, oxygen uptake was not significantly changed.

Endogenous Phase - At the end of the fourth day, sludge was harvested, washed, and allowed to metabolize endogenously in the presence and absence of ammonium sul-

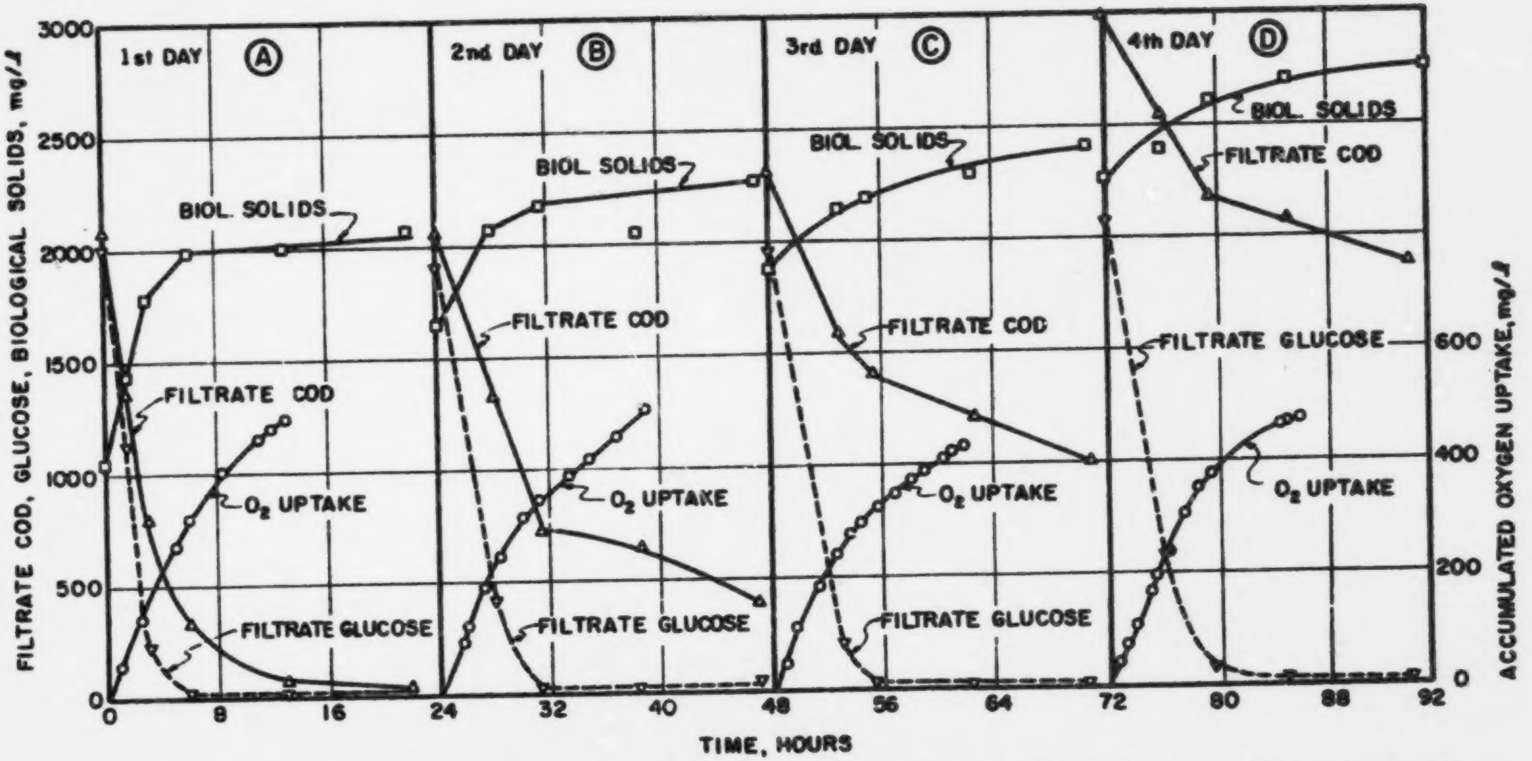

Figure 9-CHANGES IN CHARACTERISTICS OF AN ACTIVATED SLUDGE SYSTEM SUBJECTED TO SUCCESSIVE DAILY FEEDINGS OF GLUCOSE IN THE ABSENCE OF A NITROGEN SOURCE.

fate. Comparison of the changes occurring during the endogenous phase is shown in Figure 10. For the system to which ammonium ion was added, it is evident that there was a rapid decrease in sludge carbohydrate, and a corresponding increase in sludge protein, especially during the initial twelve hours. The protein and carbohydrate contents of the system to which ammonium ion was not added did not show any significant change in the 24-hour period. Endogenous respiration rate of the system supplied with ammonium sulfate was also many times higher than that of the system without nitrogen. The filtrate COD remained at a low level in both cases.

Refeeding Phase - The substrate removal capability of these sludges when they were again contacted with substrate in the absence of nitrogen source is shown in Figure 11. It is apparent that exposure of the sludge to ammonium sulfate during the endogenous phase had a significant effect upon its purification efficiency during subsequent feeding without nitrogen. Comparison of Figures 11-A and 9-A shows that the sludge had nearly regained the purification efficiency it exhibited under nonproliferating conditions immediately after being harvested from the basic unit. However, the system which received no nitrogen in the endogenous phase removed an insignificant amount of COD. The difference between the filtrate COD and the filtrate glucose (as detected by anthrone) was very pronounced for the system which had not been exposed to ammonium sulfate in the endogenous phase.

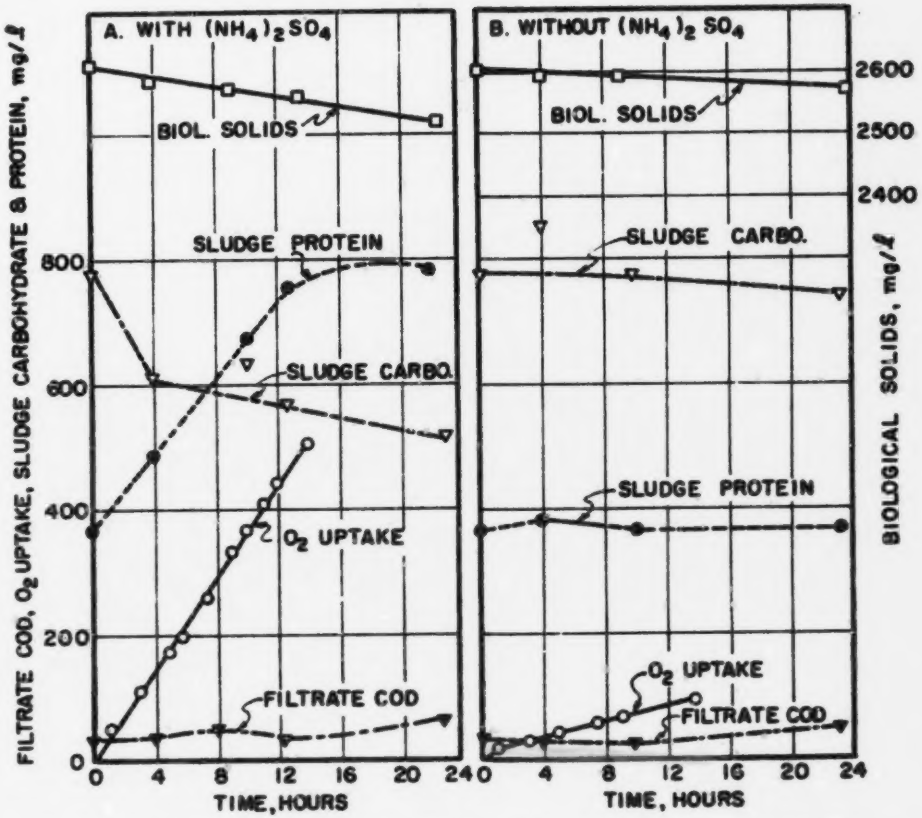

Figure 10 – COMPARISON OF CHANGES IN SLUDGE CHARACTERISTICS IN THE ENDOGENOUS PHASE WITH AND WITHOUT AMMONIUM SULPHATE FOR SLUDGE SUBJECTED PREVIOUSLY TO PROLONGED FEEDING UNDER NON-PROLIFERATING CONDITIONS.

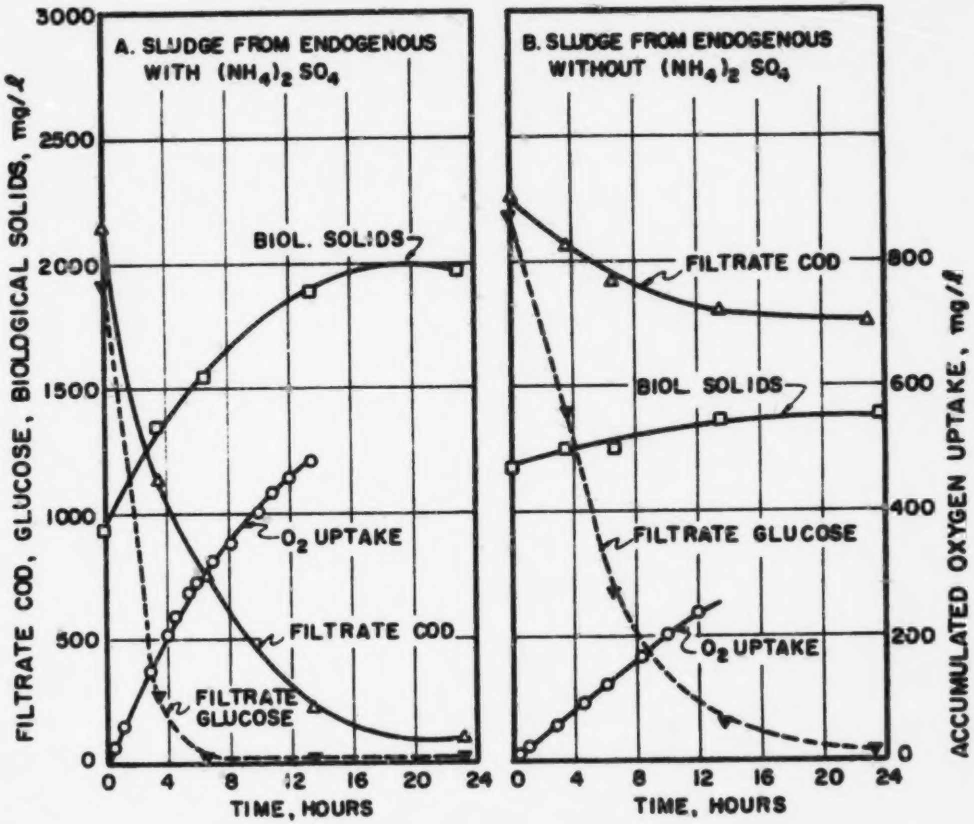

Figure 11 – COMPARISON OF SUBSTRATE REMOVAL IN THE REFEEDING PHASE FOR SLUDGE ORIGINALLY HARVESTED FROM A PROLONGED FEEDING PHASE WITHOUT NITROGEN SOURCE AND SUBJECTED TO THE ENDOGENOUS CONDITIONS INDICATED.

A summary of the changes in protein and carbohydrate of the sludge during all three phases of the long-term study is shown in Figure 12. It is seen that the carbohydrate content increased immediately after each daily feeding, and again decreased after reaching a peak. The protein content of the sludge continued to decrease throughout the first feeding phase in which no nitrogen source was added, and appeared to reach a level which was not appreciably reduced after the third day of operation. Upon addition of $(NH_4)_2SO_4$ in the endogenous phase, the protein content was again increased at the expense of carbohydrate. It is apparent that the carbohydrate content of the sludge provided the carbon source for protein synthesis.

Microscopic observation of the sludges using India ink staining technique indicated that there was no significant buildup of capsular material throughout the study. Also, the systems were at all times flocculent and exhibited rapid settling characteristics. All experiments were run at least two times; in all cases similar results were obtained.

DISCUSSION

It is evident from the results that the carbon source was effectively removed in the absence of exogenous nitrogen, and that the carbohydrate which was synthesized during the first phase was readily converted to protein during endogenous metabolism in the presence of nitrogen. This internal conversion permitted the sludge to be used again

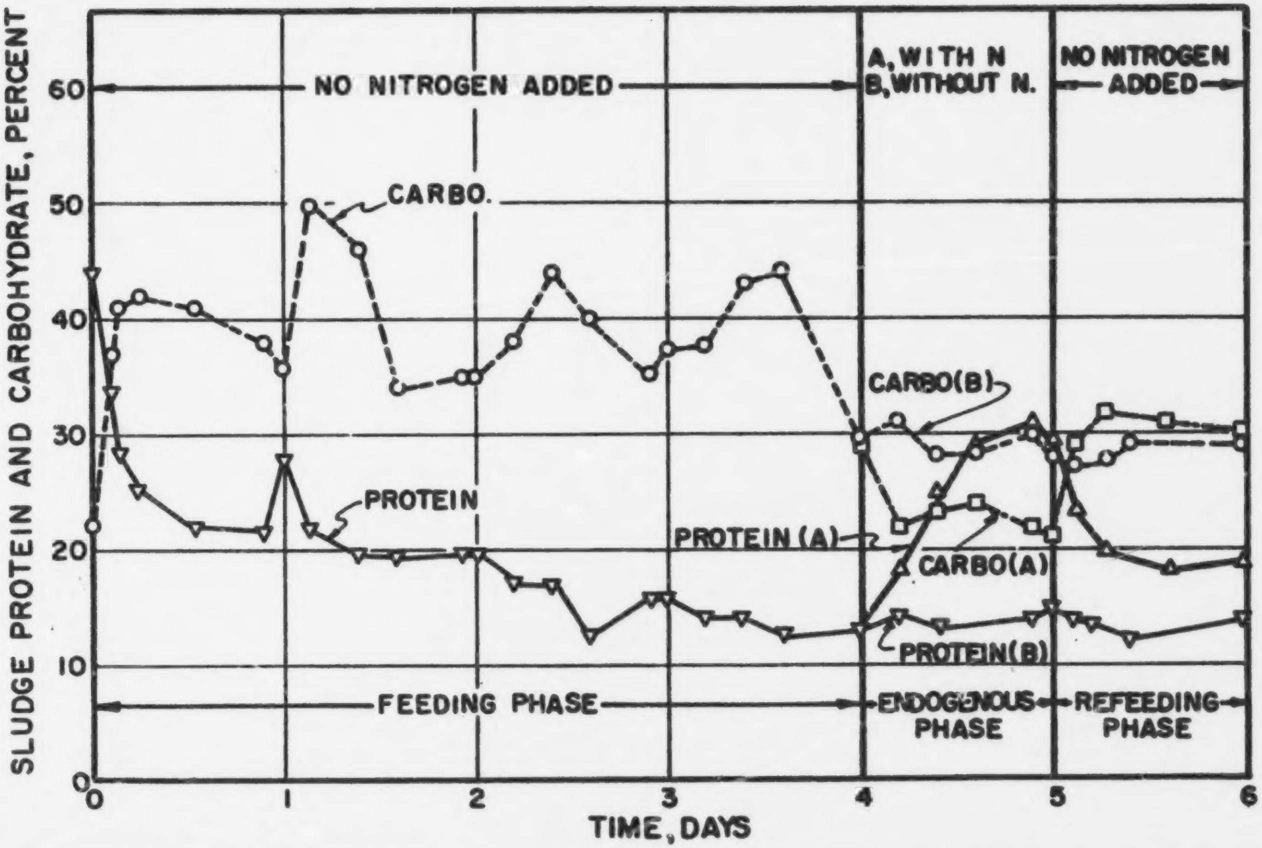

Figure 12-COMPARISON OF CHANGES IN PERCENT PROTEIN AND CARBOHYDRATE OF THE SLUDGE FOR THE CORRESPONDING SYSTEMS SHOWN IN Figures 9, 10, AND 11.

for substrate removal in the absence of nitrogen. Furthermore, even after purposely exceeding the carbohydrate-synthesizing capacity of the sludge, thereby deteriorating its substrate removal capability, the sludge could be regenerated by endogenous metabolism in the presence of nitrogen. Thus, it is seen that under the conditions herein employed, the proposed mode of operation shown in Figure 1 is a reasonable possibility.

While this type of operation does seem feasible, would effect a savings in nitrogen, and could greatly reduce the opportunity for stream enrichment, it should be approached with caution. For example, it cannot be recommended for the treatment of waste streams which exhibit a large degree of fluctuation in the structure of the carbon source. It has been previously shown that in systems subject to qualitative shock loading, a source of nitrogen is required in order that the cells may acclimate to new carbon sources(10). The range of carbon source structures which will not yield problems from the qualitative shock load is under investigation in the authors' laboratory. There is another aspect which may indicate a need for caution in employing a nonproliferating feeding phase. It was noted in these studies and in others(11)(12) that at times intermediates and/or end products are excreted into the medium during glucose removal. There is some indication from past work(12) that before these compounds can be used by the cells, an acclimation period may be needed, and for this some amount of exogenous nitrogen may be required. Conditions under which such release of metabolic products occurs are currently under investigation. It should also be noted that the success of the process shown in Figure 1 depends upon the ability to separate and control two key occurrences: first, the unbalanced synthesis of non-nitrogenous products; second, turnover of these products to protein (after separation from the mixed liquor) in an endogenous phase in the presence of nitrogen. There is no guarantee that either or both metabolic processes will occur for all carbon sources. For example, depending upon the predominating organisms and type of substrate, the products synthesized in the absence of nitrogen may not be readily available as a future energy and carbon source. Such might be the case if the product were an extracellular carbohydrate. This aspect warrants further investigation; however, in the present study the carbohydrate store was available to the organism, and it would seem likely that such would be the case for the majority of carbohydrate wastes.

The concept of utilizing a separate endogenous phase to convert storage products to protein depends upon the early synthesis of low energy-requiring products which are later channelled into protein synthesis. When the substrate itself is a carbohydrate, it may be expected that the main product synthesized will be a carbohydrate polymer, e.g., a starch or glycogen-like product. The concept put forth in these studies does not seem unrealistic, since its essence is simply to cause a totally sequential synthesis by physically separating the two processes which take place in a somewhat sequential manner in the normal growth environment of an activated sludge process wherein relatively low substrate to cell ratios obtain. Previous studies(2)(13) have shown that even in a growing system the major synthesis product, on a proportional basis, during the substrate removal period (at fairly low ratios of substrate to biological solids concentrations) is carbohydrate, and that protein is later synthesized at the expense of the carbohydrate.

It is important to emphasize the significant effect that biological solids concentration plays in determining the course of synthesis and of substrate removal. In order to observe (and make use of) the early synthesis of storage products, the system must contain relatively high concentrations of active biological solids, since the actual substrate removal cannot be made dependent upon cell replication. The use of a fairly high cell concentration is normal in activated sludge systems. While perpetuation of the process is obviously dependent upon replication of the organisms, it is apparent that the substrate removal phase is not. On the other hand, when starting with low initial solids concentrations, the early synthesis of carbohydrate undoubtedly occurs, but there are so few cells in the system that their participation is not manifested in a significant amount of substrate utilization. In this case substrate removal is actually dependent upon the presence of an exogenous nitrogen supply which is needed for channelling the early store into synthesis of protein and nucleic acids needed for replication. Thus the cell concentration in the system not only determines its kinetic characteristics, but does actually affect the gross mechanisms of substrate removal. In high solids systems almost all of the substrate can be removed in a reasonable time before appreciable amounts of protein are synthesized, whereas in low solids systems substrate removal depends upon protein synthesis leading to replication.

While the flow diagram shown in Figure 1 can be proposed for a wide range of modifications of activated sludge processes, it is related in some of its physical arrangement to the biosorption process. The results of the present study help explain the mechanisms involved in this process and offer a possible way to improve upon its operation. It is known from past work(10) that the mechanism of rapid substrate removal for various soluble carbohydrates does not involve adsorption but does entail a metabolic assimilation of the substrate. Also, as previously stated, it is known that, under growth conditions, during the substrate removal period even though carbohydrate and protein synthesis go on concurrently, there is a proportionately greater synthesis of carbohydrate than protein. The stored carbohydrate is then channelled into protein synthesis. Such an hypothesis is also supported by the work of McWhorter and Heukelekian(14) in which they have shown that there is an increase in the nitrogen content of the cells even after the biological solids production in the system has reached its maximum and the exogenous substrate has been exhausted. Due to the very short period of contact in the biosorption process, there is no opportunity for a large amount of protein synthesis in the first aerator, and the so-called regeneration or reactivating basin is simply one which provides for the conversion of the stored products to protein. Unfortunately, the great majority of the nitrogen added directly to the incoming waste stream may pass out with the effluent and may not be available where it is needed most. With proper attention to the needs for caution previously cited, addition of nitrogen to deficient wastes in the regeneration phase should enhance its more efficient use. It is emphasized that a process such as shown in Figure 1 is not necessarily intended to operate at the extremely high solids levels of biosorption, and the retention period in the feeding phase need not be as short.

The work is now being extended to other substrate systems and continuous flow operation, and to more precise determination of nitrogen requirements under this type of operation. Based upon the present study, it appears that the type of operation herein reported could be successfully applied to certain industrial wastes which are predominantly carbohydrate in nature, such as wood pulping, and sugar beet wastes.

SUMMARY AND CONCLUSIONS

The results of this study substantiate previous findings that at relatively high solids to substrate ratios the substrate is removed rapidly, and stored primarily as carbohydrate. This stored material is then converted to protein in the presence of nitrogen. These findings are the basis for a proposed flow diagram (Figure 1) for activated sludge treatment of carbohydrate wastes in which the substrate removal phase and the synthesis of nitrogenous cell components are physically separated. Nitrogen is added only to the settled sludge in an endogenous phase following substrate removal, release of the effluent, and wasting of excess sludge. Thus no nitrogen is wasted to the receiving stream. It was found that at a high solids to substrate ratio substrate was efficiently removed in the absence of nitrogen, and that aeration of this sludge with a nitrogen source restored its ability to remove substrate when again exposed to a nitrogen-free substrate. Such regeneration was possible even after prolonged exposure to nitrogen-free conditions which almost completely destroyed the substrate removal efficiency of the sludge. The proposed treatment system should be approached with caution in the case of wastes subject to qualitative shock loads, but for carbohydrate wastes of relatively constant composition a reduction in nitrogen requirement and protection of the receiving stream may be achieved.

ACKNOWLEDGEMENT

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PAPER 15

MECHANISM AND KINETICS OF SUBSTRATE UTILIZATION AT HIGH BIOLOGICAL SOLIDS CONCENTRATIONS

By

P. Krishnan and A. F. Gaudy, Jr.

This paper was presented at the 21st Purdue
Industrial Waste Conference, May, 1966.

MECHANISM AND KINETICS OF SUBSTRATE UTILIZATION AT HIGH BIOLOGICAL SOLIDS CONCENTRATIONS

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In a previous paper Rao and Gaudy(1) reported on the effect of sludge concentration on various aspects of biological activity in activated sludge, such as the relationship between rate of substrate removal and initial biological solids concentration, cell yield, and the percentage of the theoretical oxygen demand exerted at the time of substrate and COD removal. The relationship between substrate removal and initial solids concentration was found to be linear, but the value of the slope of the relationship depended not solely upon the type of substrate but upon predominating microbial species. The sludge yield varied between 48 and 82 per cent for a single substrate, glucose. Concerning the percent theoretical parameters, the results were in general accord with those of McWhorter and Heukelekian(2) with respect to COD, but not with respect to

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substrate removal as measured by a test specific for carbohydrate. A theory and experimental evidence of its validity were presented to explain the results. Since the highest biological solids concentration employed in the previous study was approximately 2000 mg/1, it was felt that further studies at higher solids concentrations were warranted. In addition, there is still much controversy over the mechanism of purification during rapid substrate removal, i.e., adsorption of soluble substrate on the floc as opposed to metabolism as the primary mechanism of rapid substrate removal. While many workers have described purification using an adsorption isotherm(3, 4, 5, 6), it is emphasized that kinetic formulation is not proof of mechanism. On the other hand, the work of other investigators supports the action of biochemical processes as the primary mechanism of rapid substrate removal(7, 8, 9, 10, 11, 12). Smallwood's(13) studies indicated that soluble substrates are removed primarily through metabolic assimilation while colloidal substances are possibly removed by adsorption. Since very rapid purification is accomplished only at high solids concentration, the present study offered an opportunity to gain further insight into this mechanistic problem, and also to investigate the degree of immediate uptake of substrate by the sludge. All of these aspects are of basic importance to more complete understanding of all types of activated sludge processes, and are particularly pertinent to the biosorption process and the applicability of a process modification recently studied by Komolrit, Goel, and Gaudy(14).

MATERIALS AND METHODS

Two batch activated sludge units were started using seed from the municipal treatment plant at Stillwater, Oklahoma. Sludges were developed on glucose and sorbitol (5000 mg/l) using the 24-hour batch feeding cycle previously described (15). After a four-week period during which flocculated cells were developed and the units came into solids balance, sludge was periodically harvested for experimentation. Sludge was centrifuged, washed twice in 0.05M potassium phosphate buffer, and resuspended in an inorganic salts medium containing distilled water and the following: $(NH_4)_2SO_4$, 2500 mg/l; $MgSO_4.7H_2O$, 500 mg/l; $MnSO_4.H_2O$, 50 mg/l; $FeCl_3.6H_2O$, 2.5 mg/l; $CaCl_2.2H_2O$, 37.5 mg/l; 1.0 M potassium phosphate buffer (pH 6.8), 60 ml/l, and tap water, 100 ml/l. Fifty ml of the suspension were then filtered (0.45 µ Millipore filter) in two portions for measurement of biological solids concentration and protein and carbohydrate content of the sludge. The chemical oxygen demand of the filtrate was determined to detect the presence of any carryover COD. To the remaining sludge suspension (2 liters) a concentrated solution of glucose or sorbitol was added to yield a concentration of approximately 2000 mg/l. In all experiments acclimated sludge was employed. The same amount of concentrated substrate was added to an equal volume of the salts medium devoid of cells. A sample was taken for analyses of COD and substrate to determine the exact concentration of carbon source present at the beginning of the experiment. This concentration, added to the carryover COD or substrate, represented the best possible measurement of the actual initial COD and substrate. At thirty seconds after adding the carbon source to the system containing the sludge, a sample was withdrawn for analysis of COD, substrate, solids concentration, and the protein and carbohydrate content of the sludge. The values of COD and substrate thus obtained were subtracted from the initial values and the difference was recorded as "immediate uptake." Thereafter samples were withdrawn each ten minutes during the first hour, and less often in succeeding hours. Because of the high solids concentration, which caused an extremely high rate of substrate removal, and because the rapidity of sampling made it impossible to process the samples immediately, special precautions were taken to stop substrate removal at the time of sampling. It was deemed undesirable to stop the reactions by chemical additives; therefore a rapid cooling procedure was adopted. Samples were withdrawn and immediately placed in pre-chilled test tubes held in an ice water bath. After adding the sample, its temperature dropped rapidly to between 0 and 3°C. After cooling for one-half hour, the sample was centrifuged, the pellet was partially resuspended, and the suspension was passed through a Millipore filter. Analyses were run on the separated system. It cannot be stated with absolute assurance that this procedure immediately and totally stopped reaction; however, the following preliminary experimental observations lend validity to its use. During rapid substrate removal, COD's were run on two equal portions of a single sample, one of which was subjected to the cooling procedure and one of which was held in an unchilled test tube for an equal period (30 minutes). The COD concentration in the chilled sample was 210 mg/l greater than in the unchilled sample. During the 30-minute period much greater reduction in COD of the aerating sample was registered (approximately 700 mg/l). These experiments indicated that while mere cessation of agitation and aeration greatly reduced COD removal, the chilling procedure greatly accelerated the cessation of reaction. Other preliminary experiments were run in which portions of identical mixed liquor samples were immediately centrifuged and filtered, and others were subjected to the cooling procedure for thirty minutes before centrifuging and filtering. In all cases COD values obtained by both methods were identical, indicating that the cooling procedure caused no more loss of COD than that which may have occurred during immediate handling of the sample.

For all experiments, oxygen uptake was also measured. A portion of the cell suspension was placed in 140-ml Warburg flasks and, after equilibration, substrate was tipped in from the flask sidearm. Initial cell and substrate concentrations were the same as those in the aerated tube from which samples were taken for cell production and substrate removal. Endogenous oxygen uptake of the sludge was also measured and the unit activity of the sludge, expressed as mg O_2/hr/gm sludge, was calculated from these data. The Warburg shaker rate and temperature were 100 oscillations per minute, and 25°C., respectively. The aeration rate and temperature in the aerated tube were 2000 cc/min/l, and 25°C.

The analyses employed were: substrate COD(16), biological solids (membrane filter technique), protein and carbohydrate content of the cells(17). The glucose carbon source was measured by either the anthrone(17) or glucostat(18) test, and sorbitol by the periodate test(19).

RESULTS

Studies on oxygen limitation in the Warburg apparatus:

Previous work had indicated that up to initial solids concentrations of 2000 mg/l, COD removal and solids production in the batch tubes and in the Warburg, at the aeration rate, shaker rate, and temperatures herein employed, were the same(20). Using COD removal and solids production rate as a guide, it was felt that oxygen utilization in the Warburg was representative of oxygen utilization in the batch tubes. Material balances calculated by the method of Gaudy and Engelbrecht(21) employing Warburg oxygen utilization, yielded fairly good recoveries. However, for the present experimentation it was proposed in some experiments to use initial solids concentrations in excess of 2000 mg/l and there was some doubt that, at these higher solids levels, 100 oscillations per minute would provide enough agitation and aeration. Preliminary experiments at higher solids levels

(5000 mg/l) in the batch tube indicated that nearly 7 mg/l DO (measured electrometrically) were present at all times.

Concurrent studies to determine COD removal were run in the Warburg apparatus and in the batch tubes at various initial solids levels. The results of two such studies are shown in Figures 1 and 2. In Figure 1, for which the initial solids concentration was 1780 mg/l, it is seen that the course of COD removal in both systems was similar. COD removal was slightly more rapid in the Warburg apparatus than in the batch tube. During the experiment, maximum oxygen uptake rate was approximately 120 mg/l/hr.

Figure 2 shows results using an initial biological solids concentration of 4990 mg/l. In this experiment the maximum rate of oxygen uptake was approximately 160 mg/l/hr. From this figure it is seen that glucose was removed slightly less rapidly in the Warburg apparatus than in the aeration tube although glucose was removed in both systems in one hour. However, COD removal was considerably slower in the Warburg flask. That is, there was evidence for the production and retarded utilization of intermediates and/or end products. Since the only difference between the systems was the mode of agitation and aeration, it would appear that at this higher initial solids level COD removal rate in the Warburg apparatus (at 100 oscillations per minute) is limited, and therefore O_2 uptake in the Warburg apparatus was not taken as an indication of the rate of oxygen uptake in the batch tubes. The results of a series of experiments like the ones shown

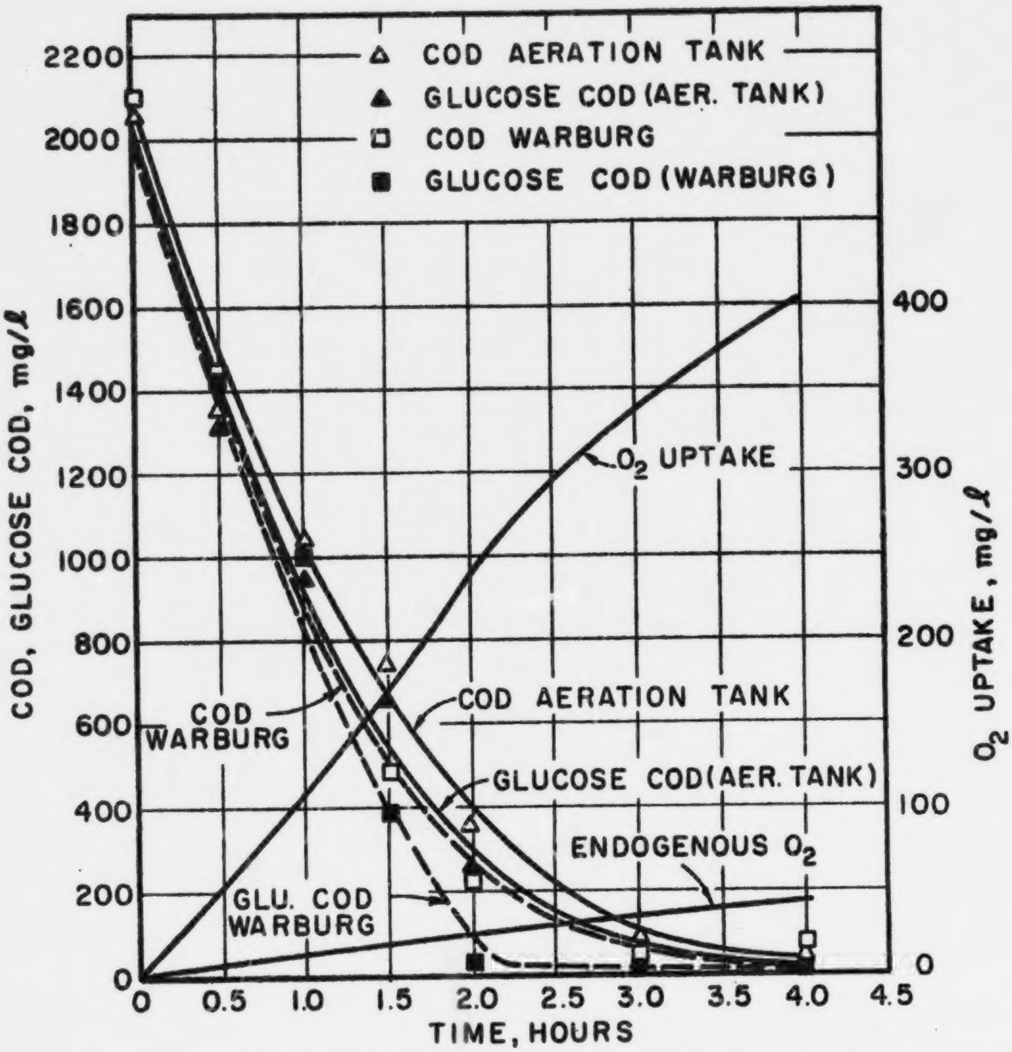

FIG. 1 UTILIZATION OF GLUCOSE IN AERATION TANK AND WARBURG APPARATUS, AT INITIAL BIOLOGICAL SOLIDS CONCENTRATION OF 1780 mg/ℓ.

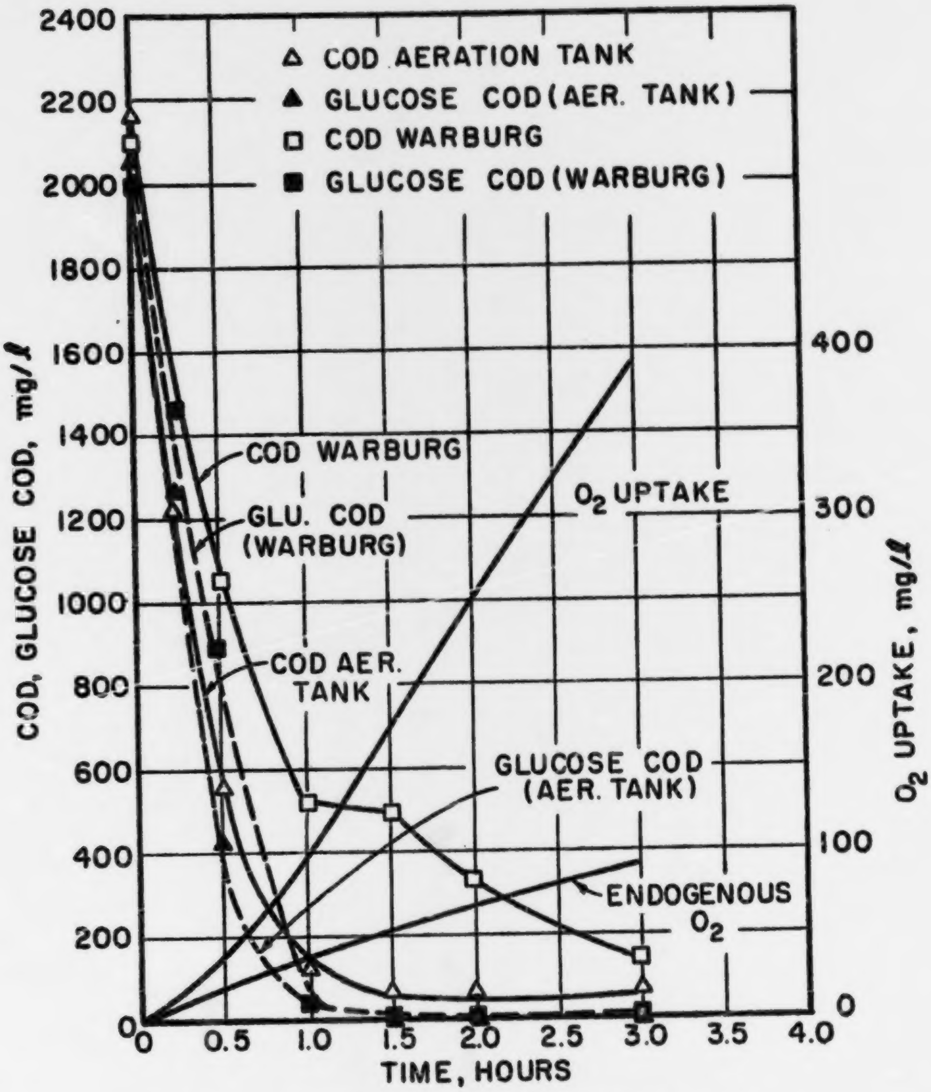

FIG. 2 UTILIZATION OF GLUCOSE IN AERATION TANK AND WARBURG APPARATUS, AT INITIAL BIOLOGICAL SOLIDS CONCENTRATION OF 4990 mg/ℓ.

in Figures 1 and 2 indicated that at initial solids concentrations of 2000 mg/1 or lower the rates of COD removal are comparable, but that they become divergent at higher initial solids concentrations. In the Warburg system (Figure 2) there was a gradual buildup of intermediates and approximately a 30-minute lag in COD removal before these metabolic products were utilized. This result offers interesting questions for investigation which are not yet resolved, i.e., if oxygen tension (shaker rate) caused the large difference between substrate and COD removal at these higher solids levels, is it because oxygen tension plays a prominent role in enzyme induction or is the difference not the result of release of intermediates of aerobic metabolism but of end products of anaerobic fermentation which may proceed concurrently with aerobic metabolism? These interesting possibilities are now under investigation in our laboratory. In any case, for the present study using solids levels above 2000 mg/1, oxygen uptake in the Warburg was not taken as indicative of oxygen utilization in the batch aerator.

Effect of solids level on mechanism and kinetics of substrate removal:

Studies on the effect of solids concentration on the mechanism and kinetics of purification using both glucose and sorbitol as carbon sources, were made in 2-liter batch activated sludge units at initial solids concentrations varying from 1500 to 4700 mg/1. Figures 3 and 4 show typical pat-

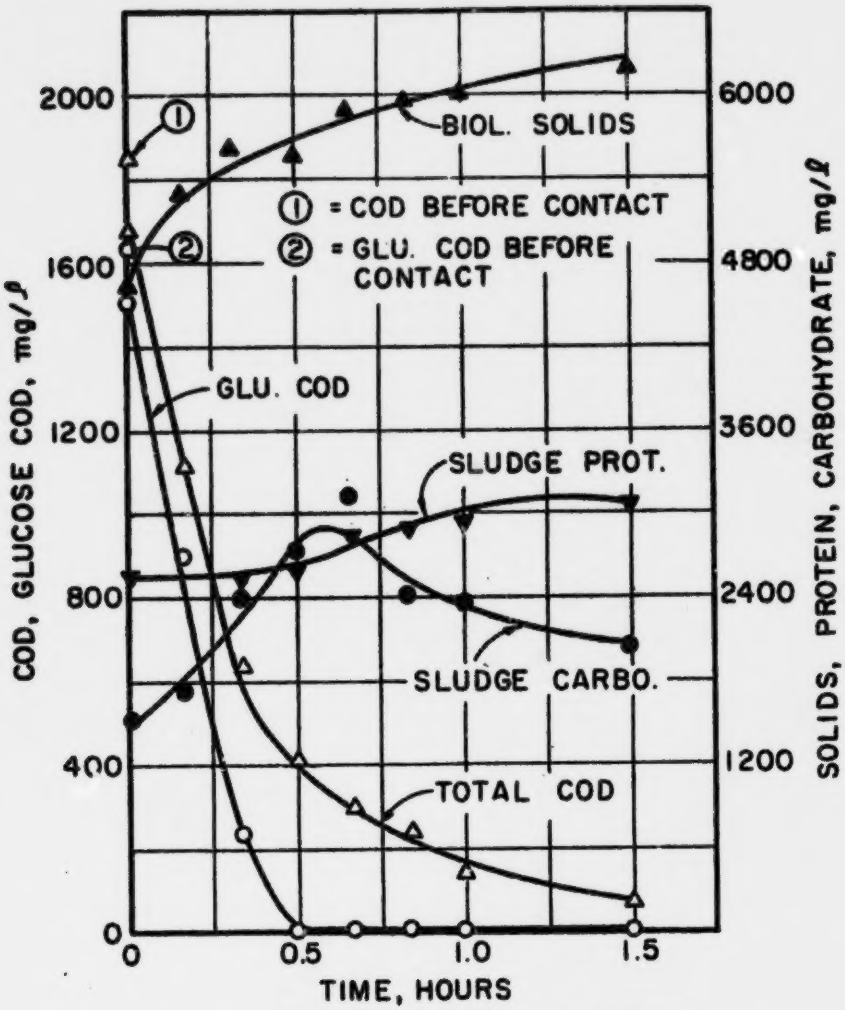

FIG. 3 METABOLISM OF GLUCOSE BY ACTIVATED SLUDGE.

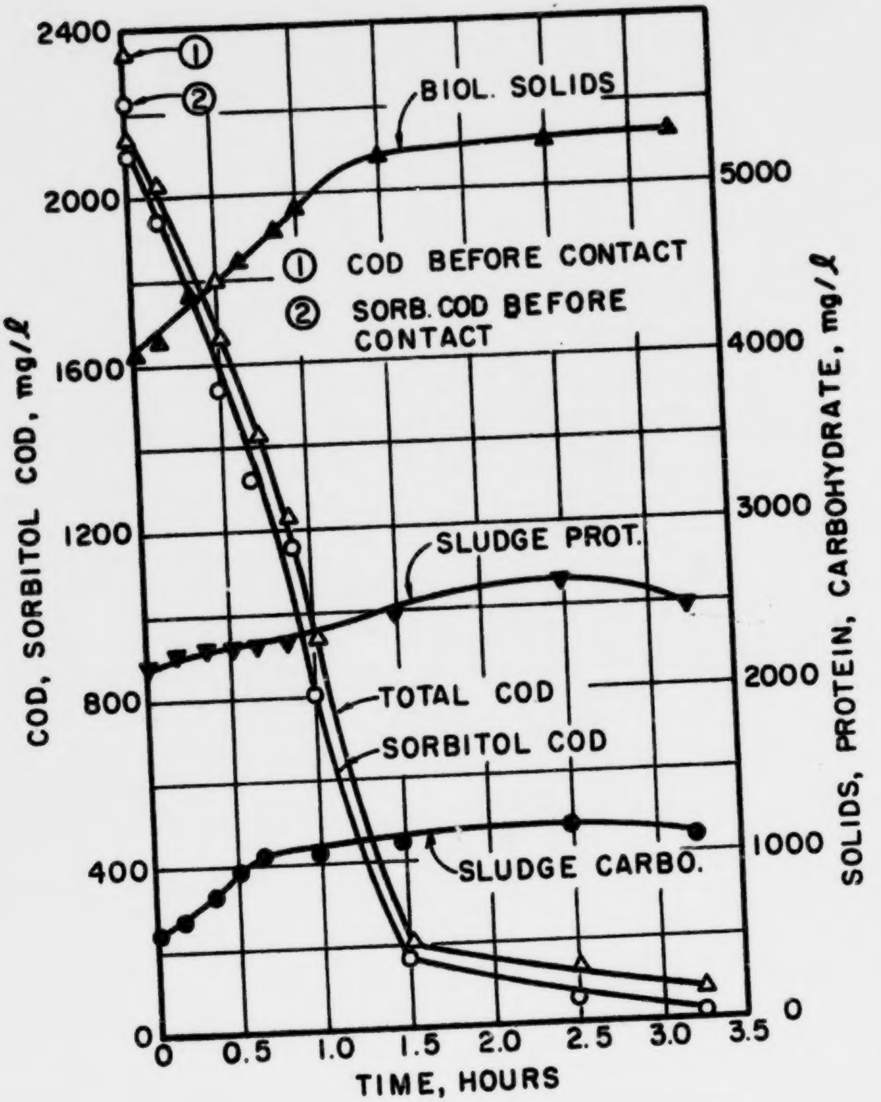

FIG. 4 METABOLISM OF SORBITOL BY ACTIVATED SLUDGE.

terns of substrate removal and sludge production at high solids concentration for glucose and sorbitol, respectively. In Figure 3 the initial values and concentrations remaining after immediate uptake (30 second sample) are plotted at 0 time. Initial COD is higher than initial glucose COD due to carry-over COD in the cell suspension, which was rather high in this experiment.

A typical experiment when sorbitol was used as substrate is shown in Figure 4. The initial solids concentration was 4117 mg/l and it is seen that the rate of sorbitol removal was rather slow compared to that for glucose (see Figure 3). This trend prevailed throughout these studies. As in the case of glucose metabolism, more of the increase in biological solids during the period of substrate removal is attributable to carbohydrate synthesis than to synthesis of protein. It is emphasized that sorbitol does not react to the anthrone test, thereby indicating that the sorbitol had to be metabolically converted to carbohydrate in order to register as an increase in carbohydrate content of the sludge.

The immediate uptake of carbon source for all experiments run at various biological solids levels is given in Table I. From these results it is apparent that, within the limits of our ability to measure it, there is immediate uptake of carbon source upon contact with the cells. Some of the immediate uptake undoubtedly represents substrate trapped in the interstices of the cell mat. The results indicate that approximately 8 to 17% of the COD is immediately taken

TABLE I

IMMEDIATE UPTAKE AND UNIT ACTIVITY OF SLUDGE AT VARYING INITIAL BIOLOGICAL SOLIDS CONCENTRATIONS

SUBSTRATE	BIOLOGICAL SOLIDS mg/ℓ	UNIT ACTIVITY mgO_2/hr/gm SOLIDS	IMMEDIATE UPTAKE		
			COD mg/ℓ	GLUCOSE mg/ℓ	% INITIAL COD
GLUCOSE	1540	11.90	280	247	11.4
	2110	5.00	336	256	13.5
	2530	8.42	177	164	7.7
	2680	5.60	403	433	17.0
	3534	12.65	432	260	16.2
	3580	5.40	324	381	12.8
	4545	4.80	330	250	15.0
	4678	9.70	175	127	10.0
SORBITOL	1594	9.42	248	160	9.8
	2501	6.35	183	140	7.4
	4117	9.60	211	197	9.4
	4712	8.90	133	140	5.3

up by the cells. It is also seen that there was no relation between immediate uptake and initial biological solids concentration.

In addition to measuring immediate uptake for the twelve experiments shown in Table I, the experiments were carried out to completion of substrate removal, and data were obtained as shown in the typical plots of Figures 3 and 4. Oxygen uptake was obtained for all experiments, but only endogenous uptake (unit activity) is reported, since we have found that above initial solids concentrations of approximately 2000 mg/l there was some doubt that O_2 uptake in the Warburg was comparable to oxygen utilization in the aeration tube.

Rao and Gaudy(1) have presented data showing the relationship between rate of solids production and rate of COD removal for solids concentrations up to 2000 mg/l. It was of interest to compare these data with those from the present experiments in which solids concentrations up to 4700 mg/l were used. This comparison is shown in Figure 5. The square symbols represent the data from the present work, whereas the small dots represent the data of Rao and Gaudy(1). The solid line represents a line of best fit for the data of Rao and Gaudy for which the slope (or cell yield) was 0.65 ± 0.17, i.e., the yield on glucose varied from 48 to 82 per cent. The data obtained in the present study would yield a slope somewhat lower than that previously obtained but all points are within the range of yields previously observed.

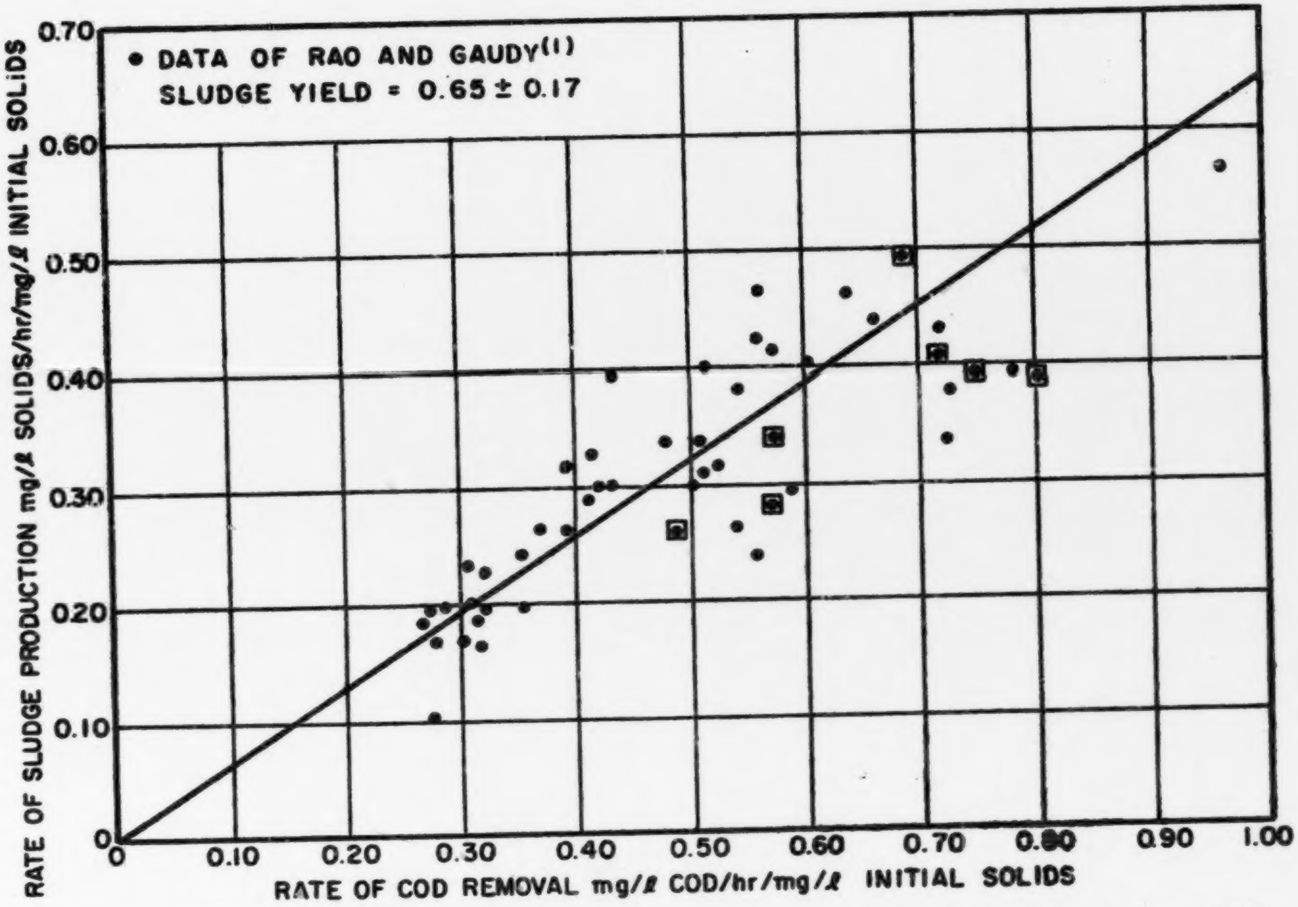

FIG. 5 RELATIONSHIP BETWEEN RATES OF SLUDGE PRODUCTION AND COD REMOVAL (SLUDGE YIELD).

Data obtained in the course of studies carried out to compare rates of substrate and COD removal and solids production in Warburg flasks and aerated tubes (see Figures 1 and 2 for typical experiments) were also used to calculate the percent theoretical O_2 uptake at the point of substrate removal. These data are compared with similar data previously presented by Rao and Gaudy$^{(1)}$ in Figure 6. It is seen that values obtained for experiments in which oxygen was not limiting fit well with the trend previously observed. The data previously presented were based on measurements of glucose removal in an aerated tube with O_2 uptake data being obtained from a portion of the same cell suspension placed in a Warburg flask. In the present experiments all data were obtained using Warburg flasks, replicate flasks being removed at frequent intervals for measurement of COD, glucose, and biological solids. The agreement between the two sets of data provides additional evidence of the equivalence of measurements made using the Warburg apparatus and aerated sludge suspensions at solids concentrations up to 2000 mg/l. Data for higher solids concentrations are also presented although these are unreliable as a measure of percent theoretical oxygen uptake due to oxygen limitation. Where oxygen is limiting, the percent theoretical uptake exerted at the point of substrate removal still decreases with increasing solids, but the decrease is less marked.

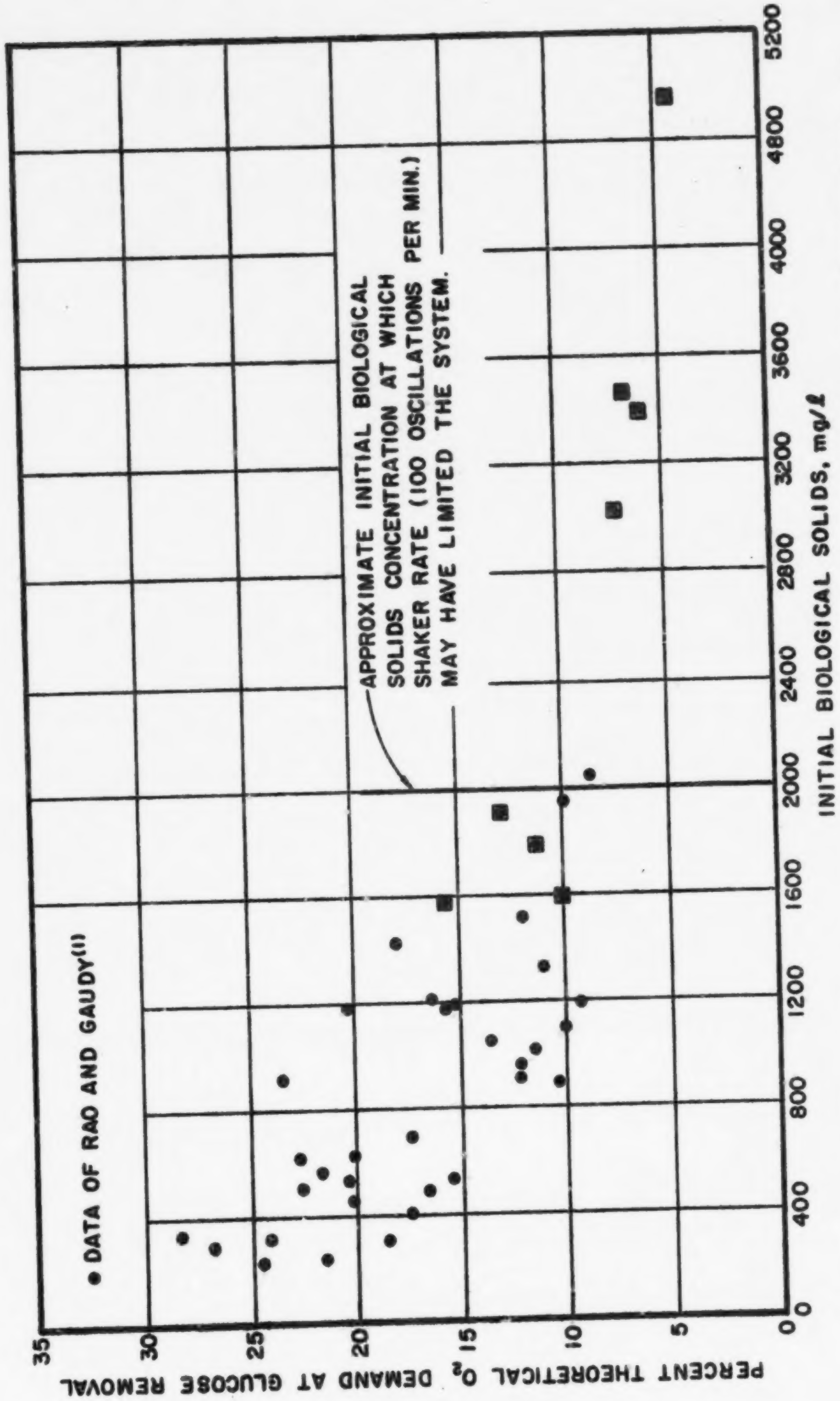

FIG. 6 EFFECT OF INITIAL BIOLOGICAL SOLIDS CONCENTRATION ON PERCENTAGE OF THEORETICAL OXYGEN DEMAND EXERTED AT TIME OF GLUCOSE REMOVAL.

In all experiments in the batch aeration tubes run at various initial biological solids levels to assess the rates of substrate removal and biological solids production, it was noted that during rapid substrate removal very little protein was synthesized, but carbohydrate production was rather high. This can be seen in the typical run shown in Figure 3. In essence, the substrate was removed in an essentially nonproliferating system, even though an ample source of exogenous nitrogen was present. Later in the run (generally after the substrate was removed), the protein content increased at the expense of the carbohydrate content of the sludge, and it appeared that the two types of synthesis were in essence sequential occurrences during the growth process. This effect has also been noted in previous studies(12)(21). In addition, previous work by Gaudy and Engelbrecht(21) and by Ramarao, Speece, and Engelbrecht(22) has shown that even in the absence of nitrogen, sludge synthesis and substrate removal could occur at practically the same rate as in systems containing an extracellular source of nitrogen. To determine whether substrate removal and carbohydrate synthesis can occur in a system in which protein synthesis is prevented by the presence of an inhibitor rather than by lack of an extracellular source of nitrogen, chloramphenicol was added to an activated sludge and the course of substrate removal and sludge production followed. These data are shown in Figure 7. It can be seen that even when protein synthesis is completely prevented, substrate (both glucose and COD) is removed at a rate comparable

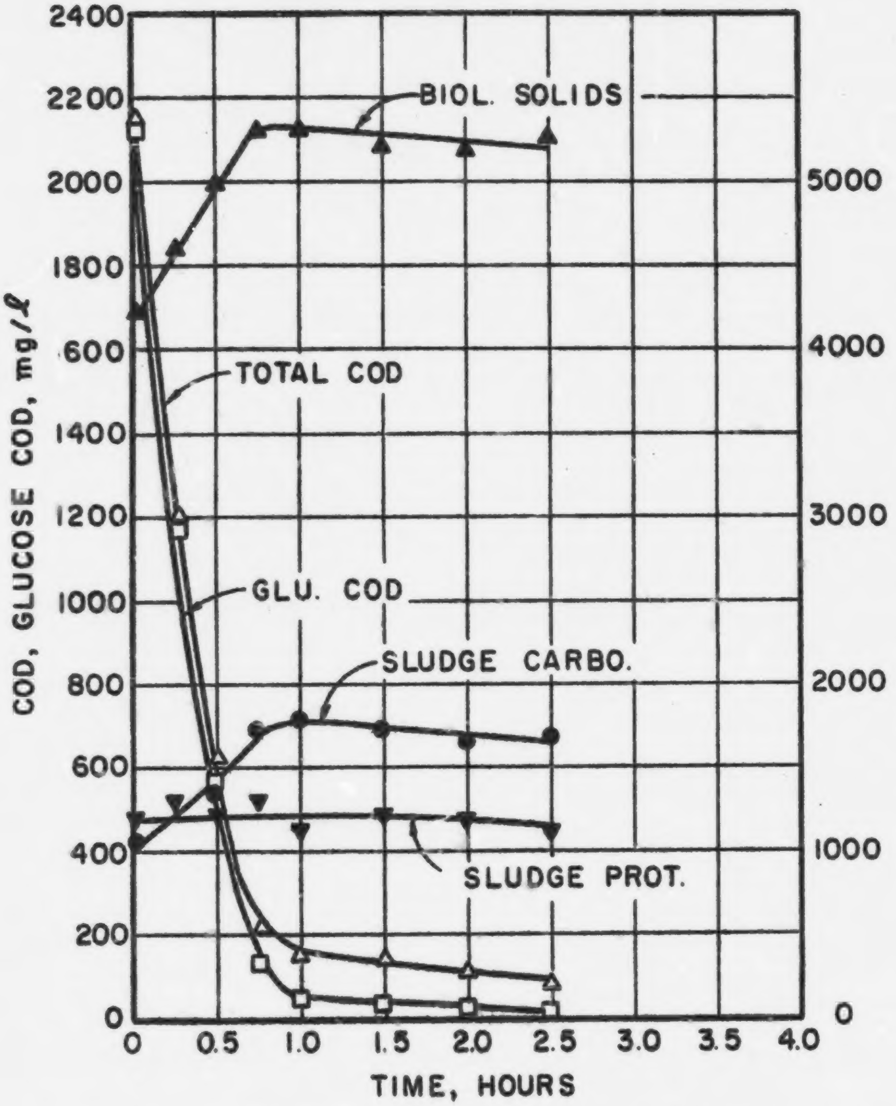

FIG. 7 EFFECT OF CHLORAMPHENICOL (50 mg/ℓ) ON GLUCOSE METABOLISM BY ACCLIMATED SLUDGE.

to that in a system where protein synthesis and cell proliferation are possible. The increase in biological solids is primarily due to synthesis of carbohydrate. In the studies using sorbitol (see Figure 4) it was seen that, like those using glucose as substrate, the major synthesis product during rapid substrate removal was carbohydrate. The results using sorbitol are particularly interesting, since they provide experimental evidence against the operation of adsorption as a primary mechanism of substrate removal.

In order to gain further insight into the mechanism of substrate removal and in particular to study further the possibility of adsorption as a primary mechanism for rapid removal of soluble substrate, it was reasoned that if adsorption played a major role, substrate should be rapidly removed in the absence of oxygen. An experiment was designed in which the sludge was agitated by bubbling nitrogen gas rather than air into the system at the rate of 2000 cc/min/l. Substrate was not added until 30 minutes after the nitrogen flow was initiated. The results, shown in Figure 8, do not support the concept of adsorption as a primary mechanism of removal of soluble substrate.

It is interesting to note that no immediate uptake was registered in this experiment. It is also seen that a considerable retardation of removal of the carbon source occurred. While the system was open to the atmosphere and opportunity was present for some surface reaeration, it can be said with certainty that the sludge was subjected to severe oxygen

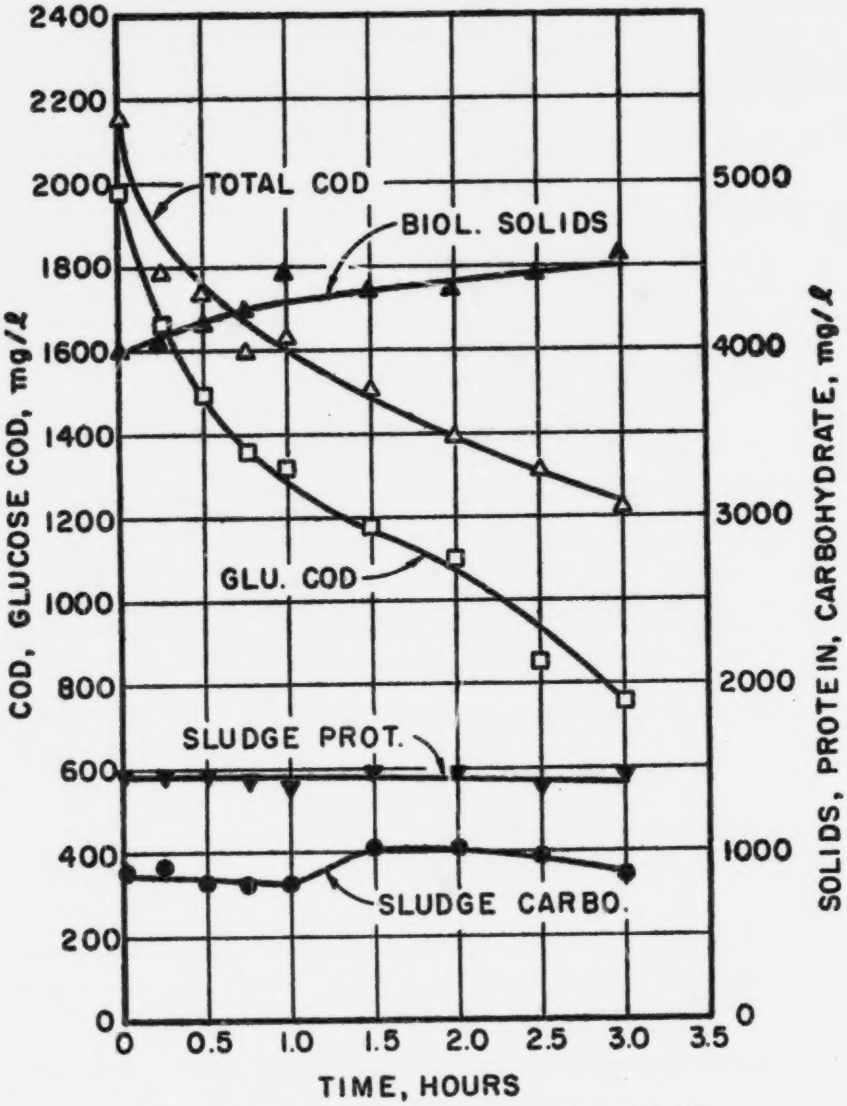

FIG. 8 METABOLISM OF GLUCOSE BY ACCLIMATED SLUDGE WITH NITROGEN AS THE AGITATING GAS.

deficiency, since only very small and erratic deflection of an electrometric probe meter was registered during the experment. The maximum instantaneous deflection noted was 3 per cent of saturation. The gradual divergence of the glucose and COD curves may be due more to a buildup of fermentation end products rather than release of intermediates of aerobic metabolism. However, it is seen that the decrease in COD is almost twice the increase in solids, i.e., the cell yield was nearly 50 per cent, which is in the range of cell yield one might expect from an aerobic system. Since the degree of aerobiosis was at best small, the possibility also exists that during metabolism volatile intermediates or end products were produced and subsequently stripped from the aeration tube. Preliminary investigations in our laboratory using well-aerated systems have shown that a portion of the volatile compounds produced during rapid metabolism of carbohydrate can be stripped from the aerator(23); this aspect is still under investigation.

DISCUSSION

The present studies are an extension at high solids concentrations of the study recently reported by Rao and Gaudy (1). It has been shown in these experiments that the sludge yield for initial solids concentrations up to 5000 mg/l is in the general range of those found at 2000 mg/l and below. The percent of the theoretical oxygen demand exerted at the point of glucose removal for solids concentrations below

2000 mg/l also agreed well with data obtained in the previous study. Since the present experiments (in which this parameter was measured) were carried out completely in the Warburg apparatus, the previous use of parallel systems in the Warburg flask and aerated tubes seems valid. It was also shown that oxygen limitation affects the rate of removal of COD (but not of glucose) in the Warburg at solids concentrations above 2000 mg/l but not below. Valid measurements of percent theoretical oxygen uptake could, therefore, not be made in the Warburg at high solids concentrations. That is, even though glucose was efficiently removed, the excretion of intermediates or end products indicated a less complete oxidation of the substrate, which would affect the oxygen demand of the system.

It has been shown in these studies (employing sorbitol and glucose) and in other studies in our laboratory that during substrate removal using relatively high initial biological solids levels, most of the substrate is channeled into carbohydrate synthesis, and that following this, protein is synthesized from the carbohydrate store, i.e., the two syntheses are observed to occur in essentially a sequential manner. As has been previously delineated by Rao and Gaudy (1) when the initial solids level is high, almost all of the substrate can be removed before an appreciable amount of protein is synthesized. It is to be expected that even when starting with low initial biological solids concentrations, carbohydrate synthesis precedes protein synthesis; however, there are so few cells in the system that the early synthesis of carbohydrate does not result in appreciable substrate removal. In this case, substrate removal actually does depend on balanced growth, i.e., replication of cells, whereas in a high solids system it does not. Therefore, the solids level affects the mechanism by which the substrate is removed. Under conditions of high solids where little or no protein is made during the period of substrate removal, it would be expected that purification would not be dependent upon a source of nitrogen. Nitrogen may actually be added after substrate has been removed, and the carbohydrate can be converted to protein. Komolrit, Goel, and Gaudy(14) have recently made use of this sequential occurrence by carrying out these processes in separate aeration vessels.

The ability of high solids systems to remove substrate in the complete absence of protein synthesis was more rigorously demonstrated by the experiment using chloramphenicol (Figure 7). In this system all protein synthesis, whether from extracellular nitrogen or from turnover of protein and pool material within the cell, was prevented. The rate of substrate removal and the pattern of initial conversion of the substrate to carbohydrate were essentially the same as for systems in which protein synthesis was not inhibited by either the presence of an inhibitor or the absence of a nitrogen source. Thus under three sets of conditions, i.e., in systems with high solids and an adequate nitrogen supply, in systems with no external nitrogen supply or in systems in in which protein synthesis is inhibited, the mechanism of removal of substrate is identical. However, only in systems where a nitrogen supply is available or in those to which nitrogen is added after substrate has been removed, is the carbohydrate subsequently converted to protein.

The relationship between the effect of solids level upon substrate removal mechanism and the effect of solids level upon the percentage of theoretical oxygen demand exerted at substrate or COD removal was delineated by Rao and Gaudy(1). As the concentration of initial solids is increased, the amount of substrate which can be removed during the initial nonproliferating phase also increases. It has been shown that if initial solids are sufficiently high, all the substrate can be removed and stored as carbohydrate before any appreciable amount of protein synthesis occurs. Since the energy required for synthesis of carbohydrate is considerably lower than that required for balanced growth, the requirement for oxygen will be correspondingly lower. In other words, since oxygen is used by the sludge in energy-producing reactions, as initial solids increases, less oxygen is required for the removal of substrate or COD. Our results are consistent with this hypothesis.

The initial conversion of substrate to carbohydrate also offers a logical explanation of the approximately zero-order kinetics of substrate and COD removal, oxygen uptake and solids production often observed with high solids systems. Since these systems function initially as nonproliferating systems, they would be expected to exhibit kinetics typical of such systems, i.e., linear or zero-order kinetics. An autocatalytic, or first-order, reaction rate cannot occur until the rate of protein synthesis reaches a sufficient level to allow a measurable increase in reaction rates due to increases in enzyme levels. While linear kinetics are, of course, always due to limitations imposed upon the system in some manner, it is not possible, without studying the mechanisms involved to draw valid conclusions as to the limiting factor. Linear kinetics of removal have been observed in systems with high solids when the carbon source, the nitrogen source and the oxygen supply were present in excess. Dissolved oxygen in aerated tanks in which the substrate was removed linearly was always 6 mg/l or above by actual measurement. Therefore, it must be concluded that the only limitation imposed upon these systems is that arising from the physiological condition of the sludge itself; i.e., sludge such as that used in these studies, or sludge returned to the aerator in an activated sludge plant, is not capable of instantaneous adjustment to a new environment or of immediate initiation of all the reactions required for balanced growth. The use of high solids, however, allows removal of substrate without all the reactions of balanced growth.

Systems with relatively high solids were also used to gain some idea of the magnitude of immediate uptake of substrate without employing tracer techniques. While the method of stopping reaction may not have provided immediate and complete cessation of substrate uptake, it was preferable to addition of a toxicant such as mercuric chloride, since such additives can cause leakage of cell material. It was observed that immediate uptake varied between 8 and 17 per cent of the initial COD, and that the amount taken up by the cells could not be correlated to cell concentration nor to substrate removal rate of the sludge during the experimental period. Immediate uptake also could not be correlated to either carbohydrate or protein content of the cells. In all cases the endogenous oxygen uptake rate of the sludge was determined and calculated as unit activity (mg O_2/hr/gm initial solids). It was found (for all but two systems) that the lower the endogenous oxygen utilization rate, the higher the immediate uptake of carbon source. If this measure of unit activity is taken as the degree of "starvation" of cells, the correlation seems reasonable. The fact that immediate uptake and unit activity did not correlate with carbohydrate content of the cells may indicate differences in the non-metabolizable carbohydrate content, e.g., cell walls and extracellular polysaccharide.

The results of the sorbitol experiments throw particular light on the question of adsorption as a primary mechanism of substrate removal. Sorbitol does not react to the anthrone test, and therefore does not register as carbohydrate. However, during substrate removal the carbohydrate content of the sludge increased rather rapidly. This finding indicates that the removal of sorbitol was the result of its biochemical conversion to carbohydrate, thus providing evidence against the adsorption mechanism.

In another experiment in which nitrogen was used as the agitating gas, the rate of substrate removal was greatly retarded and no immediate uptake was observed. It seems unreasonable to expect that a physical process such as adsorption would depend on the presence of dissolved oxygen; however, aerobic metabolism is dependent upon an adequate supply of oxygen. In this experiment the cells were suspended in buffer-salts medium and agitated with nitrogen for thirty minutes before substrate was added. This procedure insured that oxygen had been removed as completely as possible and also that the cells would be depleted of stored energy. It can be seen that this procedure completely eliminated immediate uptake of substrate, although the procedures used should affect only the rate of metabolism, not the rate of adsorption.

CONCLUSIONS

In conclusion, the results obtained in this study offer no evidence for adsorption as a primary mechanism of substrate removal, but do offer additional evidence for the purely biochemical mechanism. The results offer further proof of the hypothesis presented by Rao and Gaudy(1) for the different mechanisms of substrate removal for low and high solid systems and offer a biochemical explanation for the occurrence of linear sludge accumulation and substrate removal and the change in percent of the theoretical O_2 demand exerted at the time of substrate removal at higher solids.

ACKNOWLEDGEMENTS

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PAPER 16

KINETIC BEHAVIOR OF HETEROGENEOUS POPULATIONS IN COMPLETELY MIXED REACTORS

By

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This paper is an invited contribution to be presented at the National Meeting of the American Chemical Society, New York, September, 1966. All papers presented at this session will be submitted for publication as a symposium in Biotechnology and Bioengineering.

KINETIC BEHAVIOR OF HETEROGENEOUS POPULATIONS IN COMPLETELY MIXED REACTORS[1]

By

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SUMMARY

The kinetic behavior of heterogeneous microbial populations was studied in a continuous flow completely mixed reactor operated at various dilution rates. Glucose was used as the growth-limiting nutrient. The physiological growth parameters for cells harvested from continuous flow reactors were determined using batch experiments. It was found that the growth parameters, maximum growth rate (μ_m), saturation constant (k_s), and cell yield (Y) vary for each dilution rate, and cannot be considered as precise constants in depicting the kinetic behavior of heterogeneous populations. In addition, it was found that the yield coefficients obtained from batch experiments were always lower than those obtained from continuous flow experiments. Levels of substrate and biological solids calculated for different dilution rates using growth constants from batch experiments did not agree with the experimental values observed in steady state experiments. However, when the yield values

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[1] Presented at the national meeting, American Chemical Society, New York, September, 1966

from the continuous flow experiments were used in conjunction with batch values for μ_m and k_s, the theoretical and experimental dilute-out curves agreed fairly closely (within the range needed for engineering prediction) until the culture began to wash out of the unit. In general, the data substantiated the use of the single phase relationship between growth rate and substrate concentration described by the Monod equation, $\mu = \frac{\mu_m s}{k_s + s}$.

INTRODUCTION

Bioengineers have known for some time that the hydraulic regime established in the rectangular activated sludge aerator did not provide the plug flow conditions usually assumed in the design of such reactors. The diffusional patterns usually lie somewhere between plug flow and completely mixed conditions. Since, from an engineering standpoint, it is easier to maintain completely mixed than exact plug flow conditions, considerable attention is now being given to the formulation of equations of predictive value for designing and determining the operational parameters for fluidized bed reactors of the completely mixed type. The trend toward increasing use of completely mixed reaction vessels is also evident in microbiological research and in the fermentation industries. Indeed, work pertaining to biological reaction kinetics in completely mixed reactors in the microbiological and fermentation fields has preceded that in the water pollution control area. From a theoretical standpoint, the use of completely mixed reactors allows the system to operate in the steady rather than the transient state, and greatly simplifies the solution of mathematical expressions depicting the levels of substrate, products, and bacterial cells present in the reactor at all times.

Work with pure cultures has indicated that a steady state can be approached (1). However, in the water pollution control field heterogeneous populations are necessarily employed, and before any theoretical equation for the steady state can be applied to waste water purification, it is necessary to determine whether a steady or pseudo-steady state can be approached for such populations. Such a determination requires long term studies in order that variations in the "steady state" system parameters can be assessed and any particular kinetic model adequately tested. A model may be devised based upon the soundest biochemical theory or principle available, or assumed by any other means; however, no amount of mathematical manipulation can replace the necessary long term experimentation required to prove the applicability of the model.

One of the most useful relationships for insertion into a steady state model involves the relation between growth rate and substrate concentration. In the waste water control field there are two major concepts presently held concerning the relationship between growth rate and substrate concentration. The first may be characterized as the single phase continuous function of decreasing slope predicted according to the Monod equation (2), and the second involves the two-phase linear relationship arising from the work of Garrett and Sawyer (3).

Wilson (4) has compared these kinetic models and concluded that there was little support for the two-phase concept. However, he felt that the data used were not precise and he drew no definite conclusions as to the applicability of either model. Both models have been supported by various workers in the pollution control field. Several modifications of the Monod continuous function have also been proposed (5, 6, 7). The work reported herein is a portion of a three-year study designed to determine the extent to which a steady state can be maintained for heterogeneous populations, to obtain estimates of growth constants based upon statistical analysis of large numbers of measurements, and to use these average constants to evaluate the applicability of several proposed kinetic models to the completely mixed activated sludge process. The models investigated are briefly described below.

THEORETICAL CONSIDERATIONS

Completely mixed continuous flow biological reactors are operated on the principle that a steady state equilibrium can be maintained between the rate at which nutrients are supplied and the rate of bacterial growth; i.e., the logarithmic growth rate can be hydraulically controlled if other operational conditions are satisfied.

The theory and operation of continuous cultures has been reviewed by several authors (1, 8, 9, 10), and will be discussed only briefly here. The growth rate of organisms in the logarithmic phase may be expressed as follows:

$$\frac{dx}{dt} = \mu x \tag{1}$$

where μ is the specific growth rate, and x is the concentration of bacterial cells, or of biological solids. In order to construct a kinetic model from the basic equation it is desirable to correlate the specific growth rate to the concentration of some limiting nutrilite that exhibits an effect on the growth rate. In the biological treatment of organic waste, the limiting nutrient is usually the carbon source. The equation proposed by Monod to describe the relationship between growth rate and concentration of limiting nutrient is similar in form to the Michaelis-Menten equation, which describes the kinetics of enzyme reactions, and may be stated as follows:

$$\mu = \mu_m \left(\frac{s}{k_s + s} \right) \tag{2}$$

where μ_m is the maximum value of μ and is considered as a system constant for a particular organism under the given operational conditions, k_s may be termed the saturation constant or the substrate concentration where $\mu = \mu_m/2$, and s is the concentration of limiting nutrient or growth factor. It can be seen from this equation that the specific growth rate (μ) does not vary linearly with concentration of limiting nutrient up to attainment of the maximum growth rate.

Schulze has presented the results of studies using Escherichia coli which support the use of a modification of Monod's equation proposed by Teissier (5). The relation proposed by Teissier is:

$$\mu = \mu_m \left[1 - e^{-S/k_s}\right] \qquad (3)$$

Schulze has also applied this equation to operational data collected from waste water treatment plants (6), and has concluded that it may be used to predict the performance of prototype activated sludge plants.

Moser (7) has provided theoretical arguments indicating that the exponent of the limiting nutrient is not necessarily unity. He therefore has proposed the following modification:

$$\mu = \mu_m \left[\frac{1}{1 + k_s^{\kappa} S^{-\kappa}}\right] \qquad (4)$$

In this equation κ is a system constant which can be determined experimentally.

All three equations require the determination of various constants, e.g., μ_m, k_s, and κ. In devising a kinetic model of value in design and operation, it is also desirable to determine the proportion of substrate which is diverted into cell or biological solids production. This relationship is usually expressed as follows:

$$\frac{dx}{dt} = -Y\left(\frac{ds}{dt}\right) \qquad (5)$$

where Y is the yield coefficient and is considered to be a system constant. It is important to note, however, that the work of Rao and Gaudy (11), Hetling, et al. (12), and Marr, et al. (13) have shown that the yield coefficient is not necessarily a true constant either for pure cultures or for heterogeneous populations.

After establishing a mechanistic model relating the growth parameters considered important, the equations can be transferred readily to the steady state. For example, the rate of change in cell concentration in a completely mixed continuous flow reactor can be expressed as follows:

$$\frac{dx}{dt} = \mu x - Dx \tag{6}$$

In the steady state the rate of change in cell concentration with time is 0, and the relationship reduces to the following:

$$\mu = D = \mu_m \left(\frac{s}{k_s + s} \right) \tag{7}$$

where s refers to the concentration of substrate in the reactor. It is seen from this equation that the specific growth rate, μ, is equal to the dilution rate D, and therefore is subject to hydraulic control. The dilution rate is numerically equal to the reciprocal of the mean residence time of the fluid in the reactor. The above equation can be solved for s, leading to the following expression:

$$s = k_s \cdot \frac{D}{\mu_m - D} \tag{8}$$

The rate of change in the substrate concentration in the reactor can be equated to the rate of feeding the substrate minus the rate at which the substrate is diluted out of the system in the effluent line and the rate at which the substrate is consumed by the organisms in the reactor. This balance may be expressed as follows:

$$\frac{ds}{dt} = Ds_i - Ds - \mu \frac{x}{Y} \tag{9}$$

Where s_i is the influent substrate concentration. In the steady state, $\frac{ds}{dt} = 0$, and the steady state concentration of cells can be expressed as follows:

$$x = Y\ (s_i - s) \tag{10}$$

Using equations (8) and (10), the steady state concentrations of biological solids and substrate can be calculated for any dilution rate provided the growth constants μ_m, k_s and Y are evaluated accurately and provided these "constants" are true system constants.

The brief development of the kinetic equations given above and the various substitutions which can be made for the value of the growth rate constant were derived for completely mixed reactors of the once-through type; however, the equations can be modified readily to include cell recycle. Experiments in which sludge recycle was included will be reported in a future publication.

In the present report some complicating but rather minor factors have not been considered; for example, no consideration was given to the possibility of exogenous substrate being used for cell maintenance, nor was any consideration given to the loss of cell mass due to the possibility of endogenous respiration occurring concurrently with the utilization of exogenous substrate or to death and lysis of cells. These factors may or may not be worth consideration, depending upon the type of system employed. For a once-through reactor for which the cell concentration is regulated solely by the concentration of incoming substrate and yield coefficients, such possible refinements in the kinetic model do not seem necessary. In systems employing a relatively high recycle ratio to maintain a solids level considerably greater than can be supported in active growth by the available substrate, these factors may assume greater importance. Models including these terms will be considered in connection with data to be reported for such systems.

MATERIALS AND METHODS

The continuous flow completely mixed reactor used in these studies is shown in Figure 1. The reactor (B) and the settling tank (C) were fabricated from 4-inch and 6-inch diameter Pyrex glass tubing, respectively. The reaction vessel has a capacity of 2.5 liters; the overflow weir consists of the entire circumference of the upper edge of the 4-inch diameter cylinder; the effluent launder is formed from a section of 6-inch diameter glass tubing. The feed solution is pumped from reservoir A to the reactor and intimately mixed by diffused aeration. The effluent flows by gravity to the settling tank C. In all experiments, the air flow rate was maintained at 1.6 liters/min per liter of reaction fluid, and temperature in the reactor was maintained at $25 \pm 2^{\circ}C$. For all experiments reported herein, the carbon source consisted of 1000 mg/l glucose. The composition of the synthetic waste employed is shown in Table I. Twenty liters of synthetic waste were prepared from concentrated stock solutions in the pre-sterilized reservoir. It was found necessary to sterilize the feed lines periodically to prevent

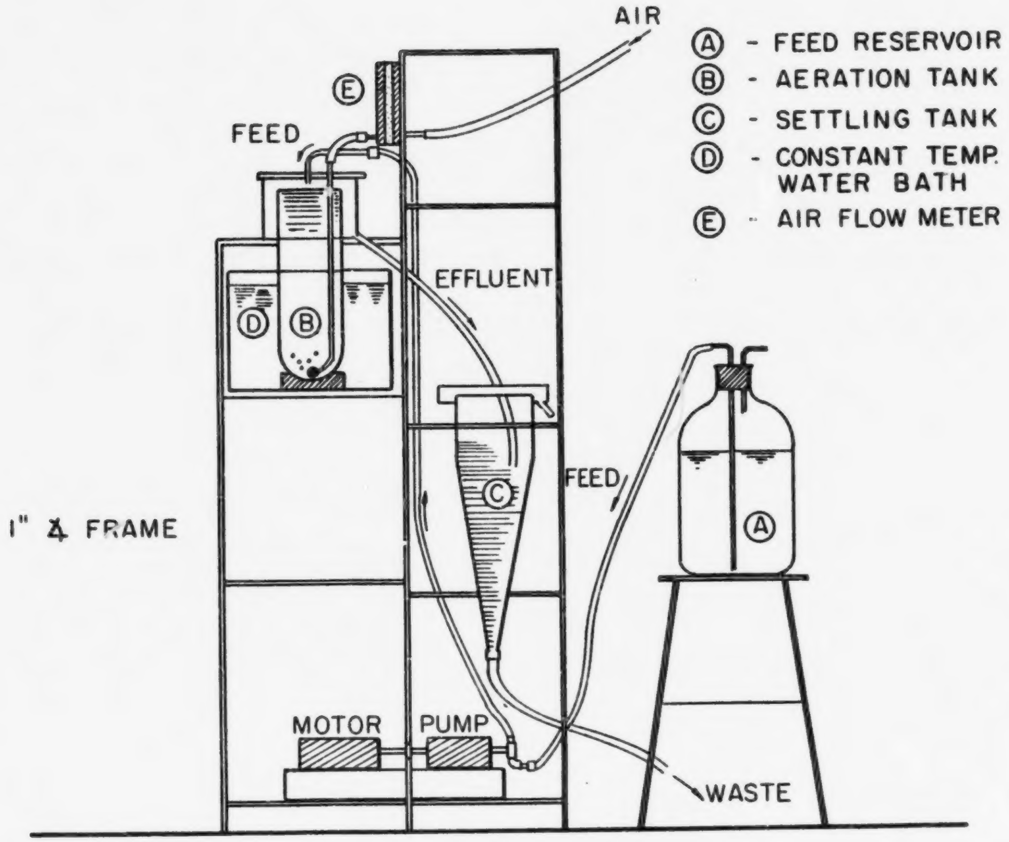
AIR
Ⓐ - FEED RESERVOIR
Ⓑ - AERATION TANK
Ⓒ - SETTLING TANK
Ⓓ - CONSTANT TEMP. WATER BATH
Ⓔ - AIR FLOW METER
Ⓔ
FEED
EFFLUENT
Ⓓ
Ⓑ
Ⓒ
FEED
Ⓐ
1" ∠ FRAME
MOTOR
PUMP
WASTE

Fig. 1 SIDE VIEW OF COMPLETELY MIXED CONTINUOUS FLOW ACTIVATED SLUDGE UNIT USED IN LABORATORY STUDIES.

back-contamination in the feed reservoir. Feed lines were sterilized by pumping a 1% Clorox solution through the lines, followed by several hours of passage of fresh water. In order to maintain continuous operation of the units, it was necessary to employ duplicate pumps and feed lines.

TABLE I

COMPOSITION OF MEDIUM FOR 1000 mg/l GLUCOSE AS GROWTH-LIMITING FACTOR

Constituents	Concentration	
Glucose	1000	mg/l
Ammonium Sulfate $(NH_4)_2SO_4$	500	mg/l
Magnesium Sulfate $MgSO_4 \cdot 7H_2O$	100	mg/l
Ferric Chloride $FeCl_3 \cdot 6H_2O$	0.50	mg/l
Calcium Chloride $CaCl_2$	7.50	mg/l
KH_2PO_4	527.00	mg/l
K_2HPO_4	1070.00	mg/l
Tap Water	100.00	ml/l

For studies at any particular dilution rate, the reactor was filled with synthetic medium and seeded with a heterogeneous population obtained from the primary effluent of the Stillwater municipal treatment plant. The system was operated in batch for several hours, and then pumping was begun and the system was allowed to come into "steady state" equilibrium. The system was considered to have approached the steady state condition when optical density and concentration of substrate in the effluent had become essentially constant. Throughout all experiments, the temperature, pH, and dissolved oxygen were recorded. Dissolved oxygen was measured electrometrically using a Jarrell-Ash dissolved oxygen meter. The major system parameters which were measured included biological solids concentration, as measured by the membrane filter technique, and chemical oxygen demand (COD) in accordance with Standard Methods (14), and the carbohydrate concentration as measured by the anthrone test using modifications previously described (15, 16). COD and carbohydrate measurements were made on filtered effluent. Daily (or more frequent) analyses were made until a sufficient amount of data had been collected to make an adequate statistical estimation of the steady state parameters.

Prior to experimentation in these continuous flow units, the system was checked for complete mixing with respect to substrate, using the procedure described previously by Komolrit and Gaudy (17). Complete mixing with respect to cell concentration was determined by periodically checking the equality of cell concentrations in the reactor and in the effluent. During prolonged experimentation at any particular dilution rate, samples of the reaction liquor were harvested, and separate batch studies were conducted to determine the values of μ_m, k_s, and Y. The batch studies were conducted in shaker flasks. In these studies the yield coefficients (Y_B) were measured during the exponential phase of growth, and this parameter is reported as milligrams dry weight of biological solids produced per milligram of COD utilized. The growth parameters obtained from the batch experiments were used in an attempt to predict the steady state values of biological solids, COD, and substrate concentration in the continuous flow reactor. Yield values (Y_C) were also calculated from the steady state values of substrate consumed and cell concentration maintained in the reactor.

RESULTS

Since the conduct of the research required massive experimental effort and the analysis of many samples for experiments conducted at various dilution rates, the presentation of all individual results would lead to an excessively prolonged and somewhat repetitive report. In the interests of brevity, results typical of individual experiments are presented graphically and summary tables showing the average values for various parameters are given.

Figure 2 shows the variation in the specific growth rate, μ, with substrate concentration, s, for one of the batch experiments using cells harvested from the completely mixed reactor during a time when the continuous flow apparatus was being operated at a dilution rate of 0.33 hrs^{-1} (detention time, 3 hrs). The concentration of biological solids was obtained by measuring optical density at 540 mμ and converting to mg/l biological solids using a previously prepared calibration curve. The values of μ at each substrate concentration were determined by measuring the slope of the straight line portion of each semilog plot and converting these values to natural logarithms. It can be seen that the specific growth rate for heterogeneous populations is dependent upon substrate concentration up to those concentrations of substrate at which μ_m is approached.

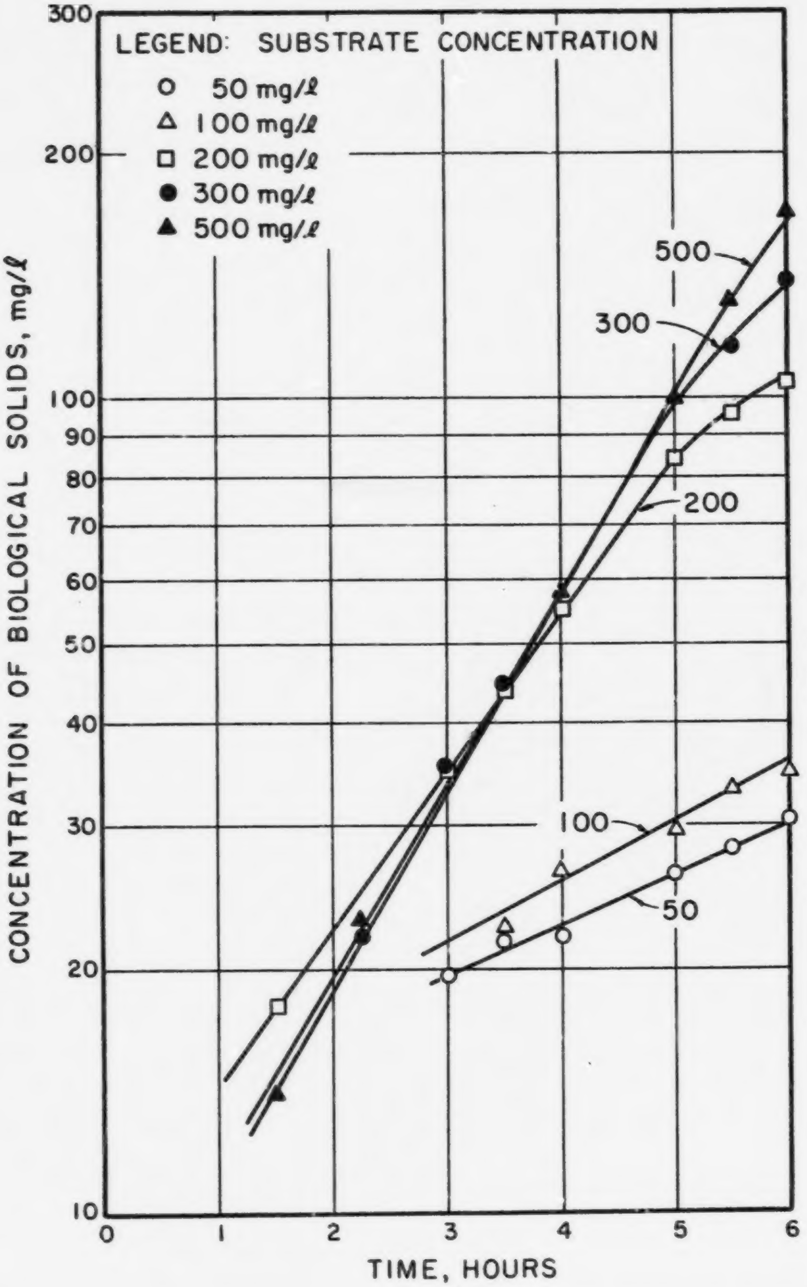

Fig. 2 GROWTH CURVES OF A MIXED CULTURE HARVESTED FROM A CONTINUOUS FLOW REACTOR OPERATING AT D = 0.33 HR^{-1}

In Figure 3 values for the specific growth rate obtained from the experiment shown in Figure 2 and from another batch experiment using cells harvested from the continuous flow apparatus at the same dilution rate are plotted against substrate concentration. The heavy solid line represents a curve drawn through the observed data. In Figure 3B the reciprocal values are plotted. Values of μ_m and k_s may be obtained from either Figure 3A or 3B by the customary methods; i.e., in Figure 3A, the maximum growth rate may be taken as the value of specific growth rate at which the curve through the observed data becomes asymptotic to an upper limit and k_s may be measured as that substrate concentration at which the growth rate is one-half of μ_m. Figure 3B is a reciprocal plot of the type devised by Lineweaver and Burk (18) for evaluation of constants in the Michaelis-Menten equation, wherein the values of μ_m and k_s may be determined graphically from the slope-intercept form of the equation. The values of μ_m and k_s obtained by both types of plot are shown in the figure. Curves I and II in Figure 3A are the theoretical plots for the Monod expression using constants obtained from Figures 3A and 3B, respectively. It is evident that the Lineweaver-Burk plot yields the better estimation of the growth parameters, since a better fit is obtained for calculated and observed data. Constants were also evaluated from the data for insertion into the Teissier equation as used by Schulze, and the resulting curve is shown in Figure 3A. It is seen that the Monod equation using constants evaluated from the reciprocal plot (Figure 3B) provides the best fit to the observed results.

Figure 3. Calculated and observed relationships between μ and S. Observed values were determined in batch experiments for cells harvested from a continuous flow reactor operated at $D = 0.33\ hr^{-1}$ with $S_i = 1000$ mg/l glucose. Theoretical curves were calculated using constants obtained from the observed data plotted according to Schulze (5) for the equation of Teissier and from plots A (curve I) and B (curve II) for the Monod equation.

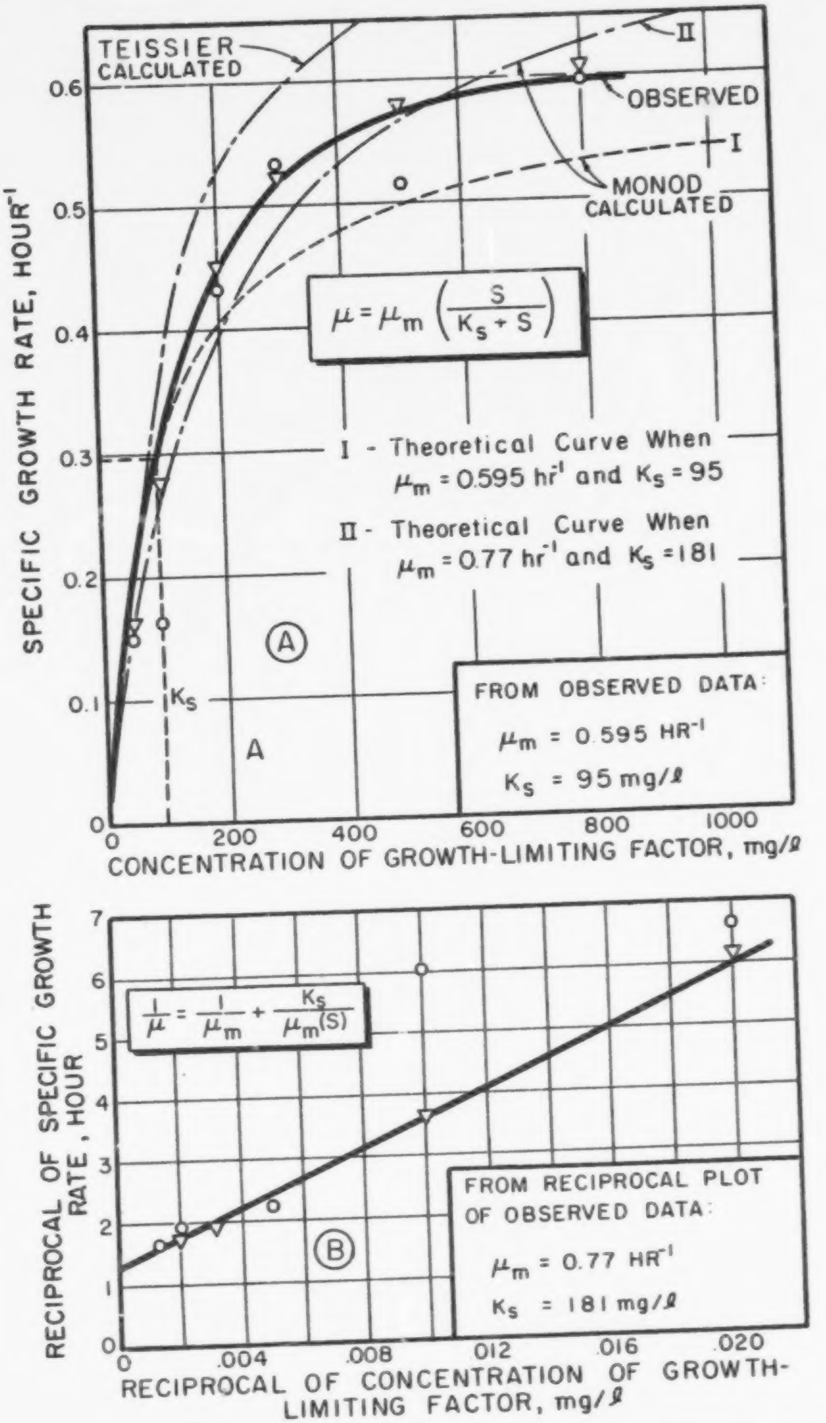

Fig. 3 CALCULATED AND OBSERVED RELATIONSHIPS BETWEEN μ AND S.

Moser's equation is plotted in Figure 4 for the various values of $\varkappa$. The plotted points are the values of specific growth rate at various substrate concentrations obtained from the data of Figure 2. When $\varkappa$ is equal to unity, the expression proposed by Moser reduces to the Monod equation. The observed data fit the theoretical curve for $\varkappa = 1$ quite well.

Figure 5 shows typical results for the variation with substrate concentration of specific growth rates for cells harvested from the continuous flow apparatus at various dilution rates. It can be seen that in general the data uphold the single phase relationship rather than the two-phase concept for which the values of specific growth rate would vary linearly with substrate up to the attainment of μ_m.

Values of the growth parameters obtained from batch experiments using cells harvested from the continuous flow reactor at various dilution rates are summarized in Table II. The values given represent the average of no less than two batch experiments at each dilution rate. Values of yield coefficients were obtained in batch (Y_B) and from the "steady state" continuous flow reactor (Y_C). It is seen that there was some variation in the specific growth rates for heterogeneous populations, and it is also interesting to note that the yield coefficients obtained from the continuous flow reactor were always higher than those obtained from batch experiments. It should be emphasized, however, that the yield coefficients obtained in continuous flow represent the average of many more determinations than those for Y_B.

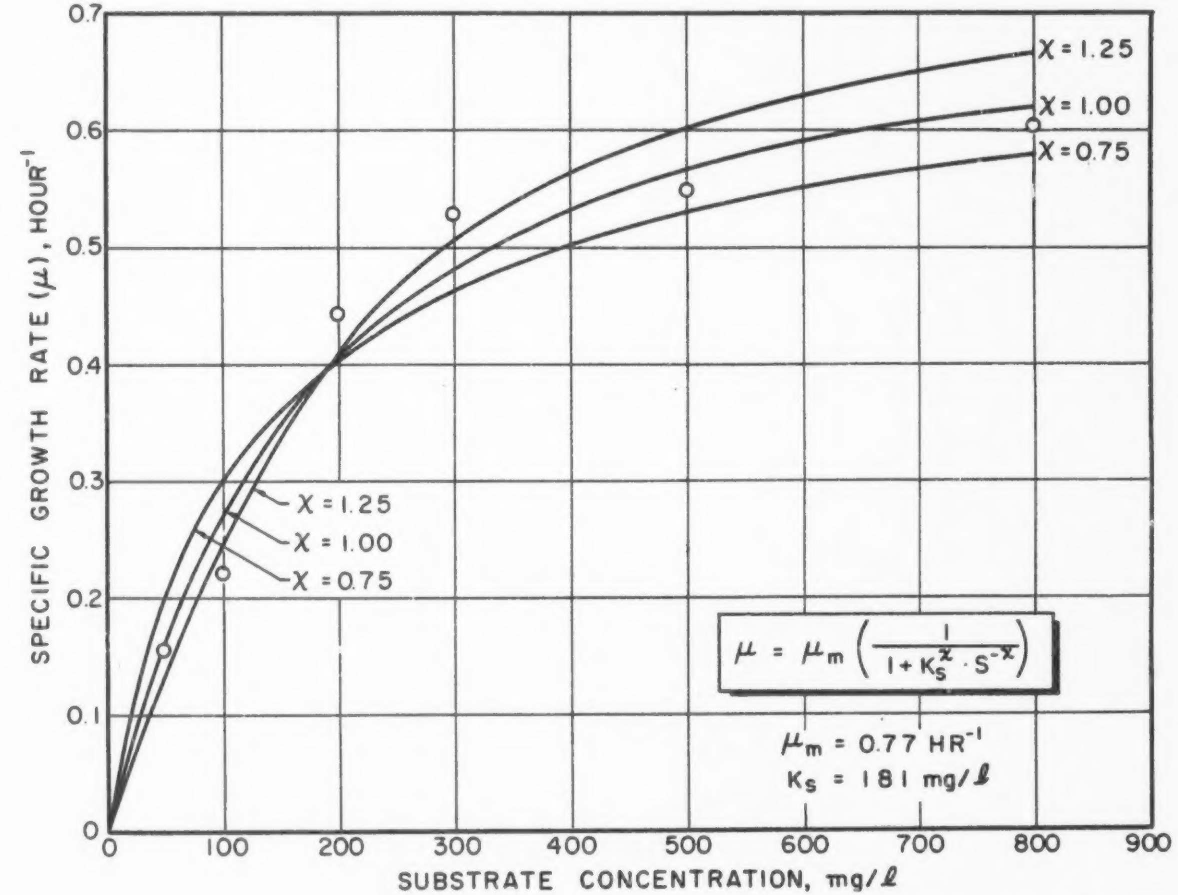

Fig. 4 RELATIONSHIP BETWEEN μ AND S ACCORDING TO THE EXPRESSION PROPOSED BY MOSER. D = 0.33 hr^{-1}, S_i = 1000mg/ℓ. PLOTTED POINTS REPRESENT OBSERVED DATA.

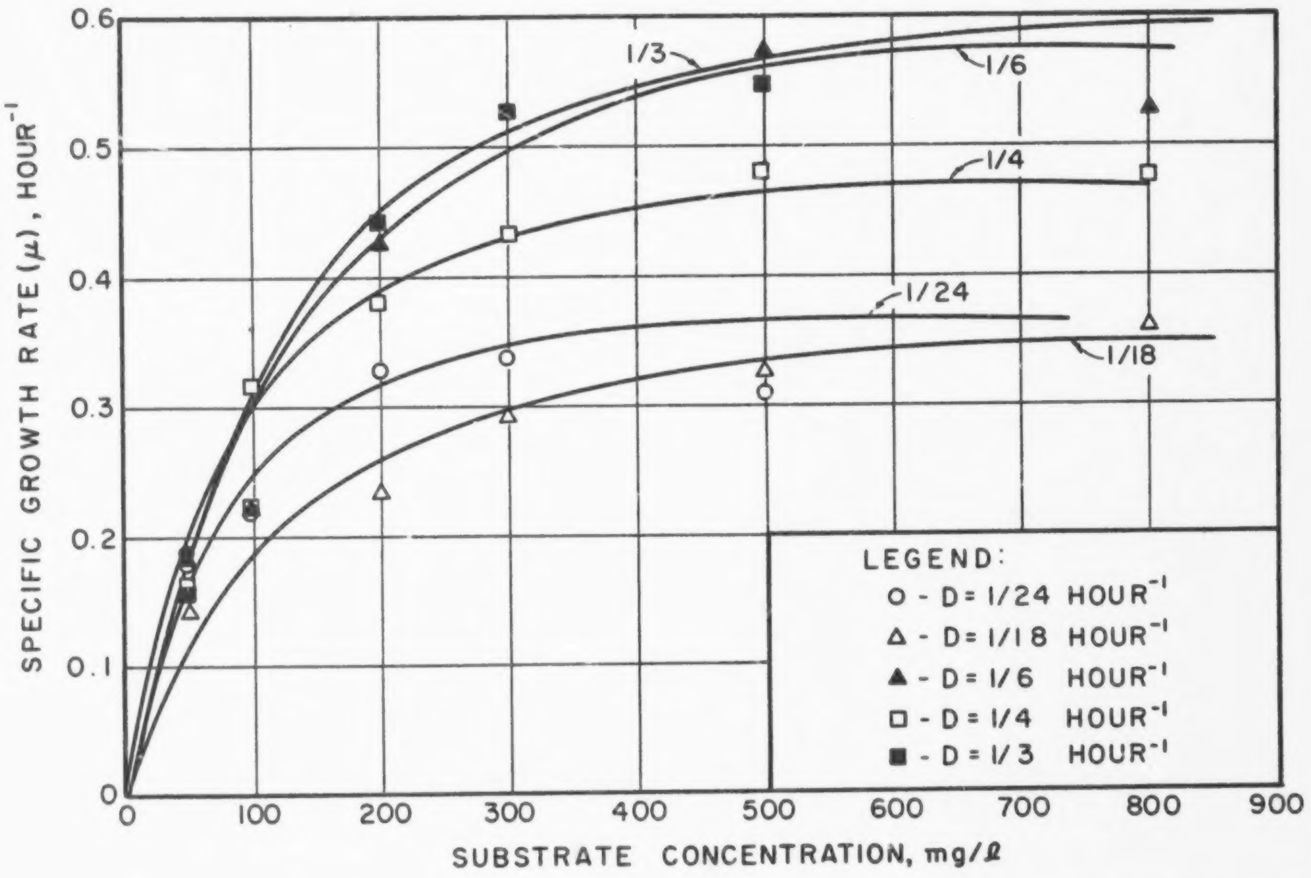

Fig. 5 RELATIONSHIP BETWEEN SPECIFIC GROWTH RATE AND CONCENTRATION OF SUBSTRATE AT DIFFERENT DILUTION RATES.

TABLE II

GROWTH PARAMETERS OBTAINED FROM BATCH EXPERIMENTS ACCORDING TO MONOD'S EQUATION

(S_i = 1000 mg/l Glucose)

Dilution Rate Hr^{-1}	Specific Growth Rate, Hr^{-1} Concentration of Substrate, mg/l						μ_{m_1}	k_{s_1}	μ_{m_2}	k_{s_2}	μ_m	k_s	Yield Coeff. Y_B	Y_C
	50	100	200	300	500	800	Hr^{-1}	mg/l	Hr^{-1}	mg/l	Hr^{-1}	mg/l	Batch	Cont.
(1)	(2)	(3)	(4)	(50	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
1/24	0.177	0.220	0.327	0.338	0.309	-	0.365	60	0.416	68	0.390	64	0.46	0.552
1/18	0.142	0.112	0.234	0.293	0.326	0.360	0.350	90	0.384	87	0.367	89	0.42	0.692
1/12	0.189	0.224	0.324	0.424	0.524	0.538	0.545	80	0.588	91	0.567	86	0.37	0.675
1/6	0.181	0.222	0.428	0.605	0.573	0.529	0.575	100	0.715	145	0.645	123	0.46	0.636
1/4	0.162	0.316	0.380	0.432	0.480	0.475	0.470	65	0.555	97	0.513	81	0.46	0.685
1/3	0.156	0.221	0.444	0.528	0.549	0.603	0.595	95	0.770	181	0.683	138	0.48	0.615
1/2	0.180	0.436	0.378	0.492	0.315	0.466	0.470	80	0.600	116	0.540	98	0.48	0.558
1/1.5	0.336	0.405	0.460	0.470	0.595	0.555	0.560	40	0.530	30	0.545	35	0.48	0.785

μ_{m_1} and k_{s_1} - obtained from the graph of μ vs. S

μ_{m_2} and k_{s_2} - obtained from the graph of $1/\mu$ vs. $1/S$

μ_m and k_s - are the mean values of μ_{m_1} and μ_{m_2}, and k_{s_1} and k_{s_2}

Figures 6 and 7 show the daily variations in "steady state" parameters during continuous operation at dilution ratios of 1/3 and 1/12 hrs^{-1}, respectively. It is emphasized that the plotted values shown in these figures do not represent all sampling points during experimentation at any particular dilution rate. In general, the points plotted are intended to show the maximum variation which was observed. The number of samples from which average values for each dilution rate were computed is approximately double those shown in the figure. The variations in incoming carbon source (feed COD) occurred due to variations in measuring the large volumes required for feeding. No serious effort was made to maintain the influent feed concentration at an exact value but, in general, the feed concentration did not vary sufficiently from 1000 mg/l to be of "shock load" proportions; the response to changes in feed concentration of shock load proportions is being studied in our laboratory in separate investigations. In these figures it is seen that essentially all of the glucose was removed from solution at both dilution rates. It is also seen that variation in effluent COD was not excessive and that the units operated in a state which approached steady state operation for both effluent parameters. However, there was considerable variation in the biological solids concentration. These variations are not attributable to random errors in sampling or in analytical technique, since many series of samples taken over short time intervals did not show such variation. A small portion of the variation may be attributed to variations in feed COD. However, experience during the

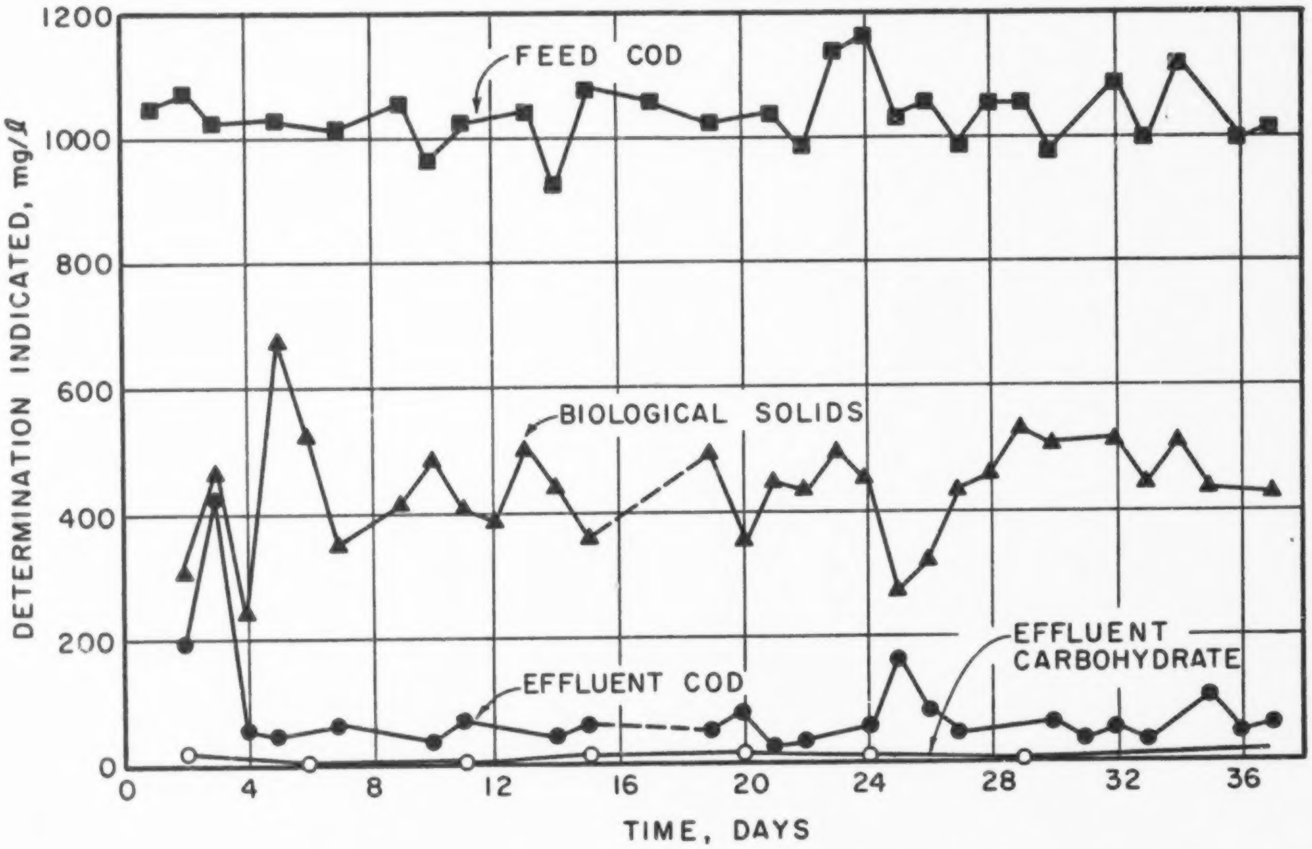

Fig. 6 "STEADY STATE" PARAMETERS AT D = 1/3 HOUR^{-1}. (S_i = 1000 mg/ℓ GLUCOSE)

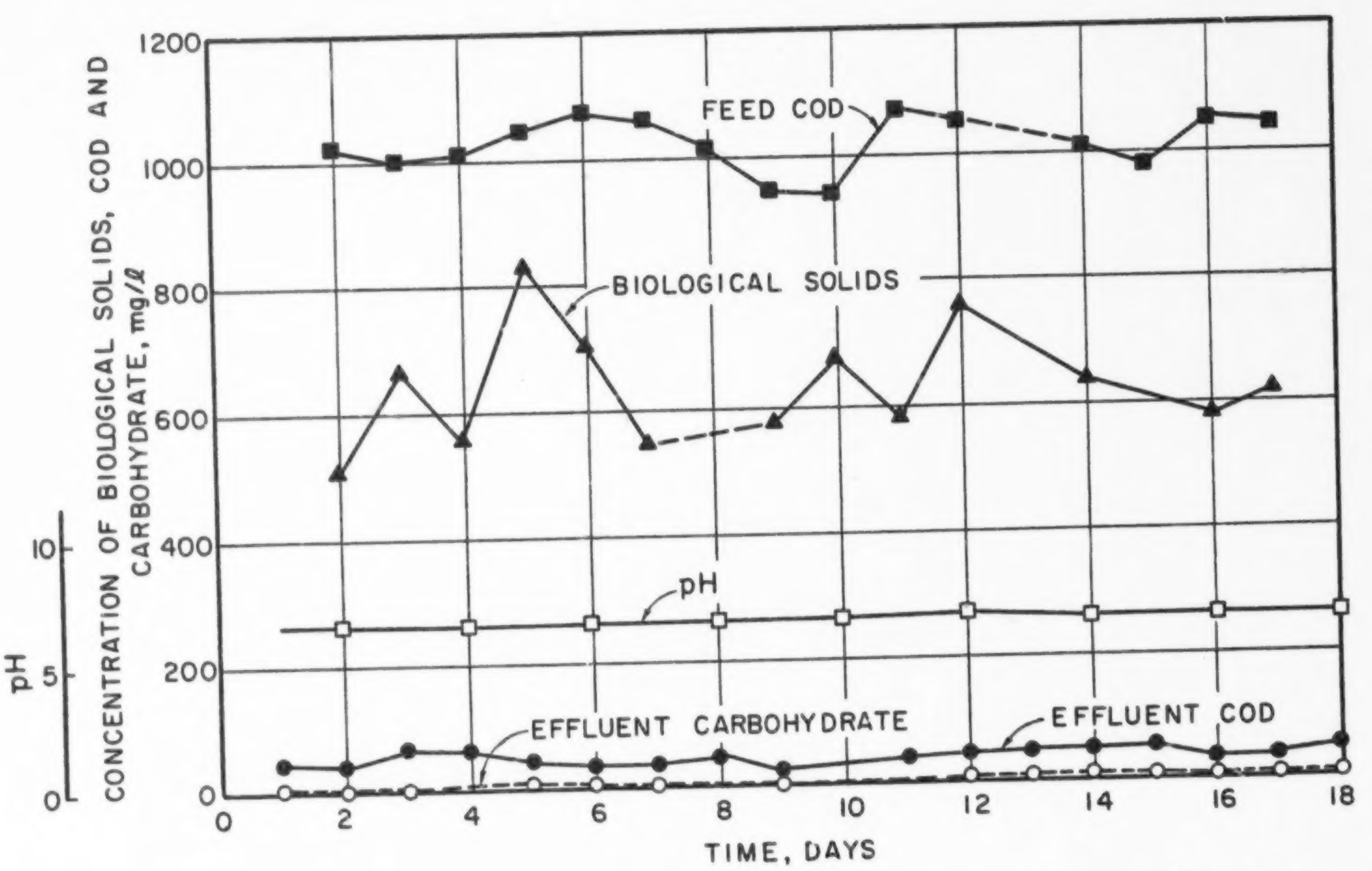

Fig. 7 "STEADY STATE" PARAMETERS AT D = 1/12 HOUR^{-1}. (S_i = 1000 mg/ℓ GLUCOSE) conduct of the research indicated that, in general, the variations in solids concentration which were observed were those which must be expected when employing heterogeneous populations, and for this reason experiments at each dilution rate were conducted for a prolonged period of time, and the various steady state levels for effluent and biological solids concentration were estimated statistically for each dilution rate.

The average values for the dilution rates under study are summarized in Table III. The series of experiments was initiated at a dilution rate of 1/24 hrs^{-1} and progressed through succeedingly higher dilution rates until dilute-out of the culture was obtained. Also shown in Table III are the specific substrate utilization rates (19), the loading factors, COD removal efficiencies, and the output rate of cells.

The average values of solids concentration, effluent COD and carbohydrate at various dilution rates are plotted in Figure 8. Using the values for growth rate constants which were determined during the study, Equations (8) and (10) may be employed to compare the observed values with those predicted by the steady state equations. Six theoretical curves (A through F) are shown for biological solids concentration. These were obtained by using various combinations of data, i.e., average values, individual values at each dilution rate, yield coefficients from batch experiments and continuous flow data, etc. (see legend, Figure 8). As shown in Figure 8, predicted curves obtained using yield coefficients from the continuous flow data fit the observed curves rather well. Curves A and B

TABLE III

STATISTICAL ESTIMATION OF THE "STEADY STATE" PARAMETERS AT DIFFERENT DILUTION RATES

(S_i - 1000 mg/1 Glucose)

Dilution Rate Hr^{-1}		$\bar{x}$ mg/1	$\bar{S}$ mg/1	$\bar{S}_i$ mg/1	$\bar{S}_c$ mg/1	Coefficient of Variance (Per Cent) x	Coefficient of Variance (Per Cent) S	Coefficient of Variance (Per Cent) S_c	$\frac{D(S_i-\bar{S})}{\bar{x}}$	Loading Factor lbs COD/ lbs SS/day	COD Removal Efficiency %	Output Rate of Cells mg/1/hr
(1)		(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
1/24	0.042	540	48	1026	5	24.3	27.9	73	.076	4.33	95.5	22.6
1/18	0.056	687	54	1047	4	33.1	23.7	75	.081	4.79	95.0	38.4
1/12	0.083	655	45	1015	4	16.6	21.7	64	.123	7.31	95.5	54.4
1/6	0.167	629	69	1058	9	45.6	21.9	46	.263	15.86	93.5	87.5
1/4	0.250	661	92	1057	8	26.2	38.5	93	.364	24.00	91.4	165
1/3	0.330	511	237	1067	214	39.5	86.2	103	.541	41.80	78.0	169
1/2	0.500	101	874	1057	698	24.0	11.9	23	.905	314	17.3	50.5
1/1.50	0.670	140	913	1091	764	71.3	11.5	24	.849	312	16.3	93.9

$\bar{x}$ - mean biological solids

$\bar{S}$ - mean effluent COD

$\bar{S}_i$ - mean influent COD

$\bar{S}_c$ - mean effluent carbohydrate

Figure 8. Dilute-out curves for biological solids and substrate at S_i = 1000 mg/l glucose. Solid lines show observed data. Curves A-F show predicted values for biological solids based on the Monod equation and using the following values for the constants μ_m, k_s and Y: Curve A, mean values of μ_m and k_s from batch data and Y_C values for each dilution rate; Curve B, individual values of μ_m, k_s and Y_C for each dilution rate; Curve C, mean values of μ_m, k_s and Y_C; Curve D, mean values of μ_m, k_s and Y_B; Curve E, individual values of μ_m, k_s and Y_B from batch studies at each dilution rate; Curve F, mean batch values of μ_m and k_s and individual values of Y_B for each dilution rate.

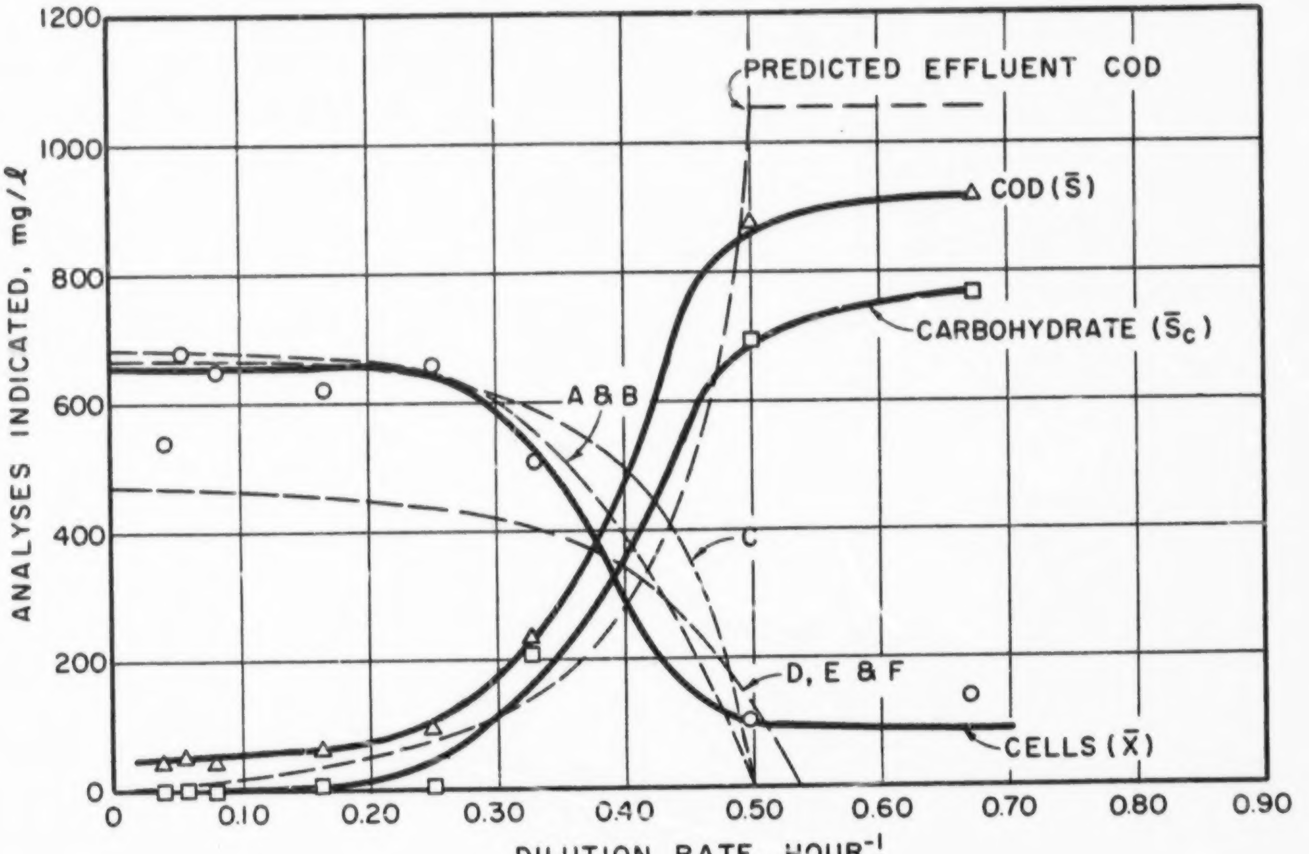

Fig. 8 DILUTE-OUT CURVES FOR BIOLOGICAL SOLIDS AND SUBSTRATE AT 1000 mg/ℓ INFLUENT GLUCOSE CONCENTRATION.

for which Y_C values at each dilution rate were combined with either average values or individual values of μ_m and k_s fit the observed data better than when the overall average Y_C value was used (curve C). In comparing curves A and B with the observed solids concentration curve, the major point of difference is the "tailing-off" at the higher dilution rate rather than the total dilute-out predicted in Equation (10). Only one curve is shown for calculated substrate concentrations since use of either individual or mean values of k_s and μ_m gave essentially identical curves.

The specific substrate utilization rate at each dilution rate (given in Table III, column 9) is plotted in Figure 9. The relationship is linear at all growth rates except those near the dilute-out region. The highest dilution rate (or growth rate) plotted is one observed beyond the point at which cells should have diluted out of the system in accordance with Equation (10).

DISCUSSION AND CONCLUSIONS

In general, the experimental results support the applicability of the Monod relationship in preference to the equation of Teissier or Moser. Also, there was no experimental evidence to support the two-phase kinetic concept in which a linear relationship between growth rate and substrate concentration would be predicted at growth-limiting concentrations of substrate. As seen from the theoretical plots of Figures 3 and 4, the curves generated by either the Monod, Teissier, or Moser

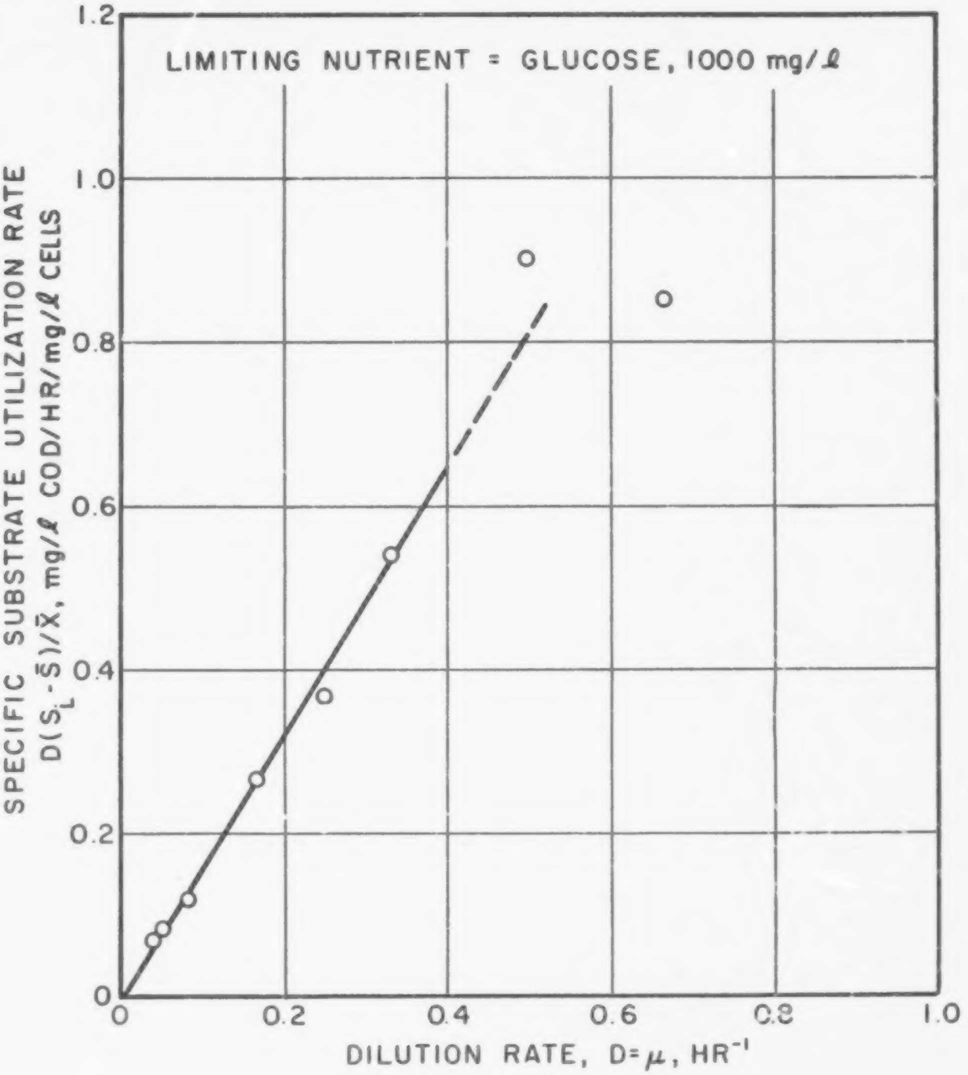

Fig. 9 RELATION BETWEEN DILUTION RATE AND SPECIFIC SUBSTRATE UTILIZATION RATE DURING "STEADY STATE" GROWTH.

equation predict a decreasing, rather than a constant, slope as μ approaches μ_m. The experimental data plotted in Figure 5 fit a curve of decreasing slope. Various aspects of both kinetic concepts have been discussed by Gaudy and Gaudy (20). It is important to note that theoretical justification of either concept is lacking, and that all the kinetic formulations which are used to relate growth rate and substrate concentration are essentially empirical expressions. Thus selection of any one model, for practical application, may be appropriately based upon a curve-fitting approach. However, it must be recognized that such an approach does not provide proof of the theoretical validity of any particular kinetic model. The results presented herein offer support for the practical application, rather than the proof, of the Monod equation in reactors employing heterogeneous populations. With regard to the choice of the two-phase or single-phase kinetic model, if an investigator bases his conclusion or selection of one type over the other on the basis of best fit of the data, it must be realized that there are situations where either concept would seem justified. For example, in Figure 10 five curves are plotted in accordance with the Monod relationship for μ_m of 0.4 hr^{-1} and k_s values varying from 10 to 150 mg/l. Turning attention to curve E, for which the curvature is slight, it can be seen that experimental data grouped around this curve might be plotted as a straight line up to some value of μ_m below the true value, 0.4. Also, considering experimental data which might be grouped around curve A, it can be seen that this curve might easily be fitted to a straight

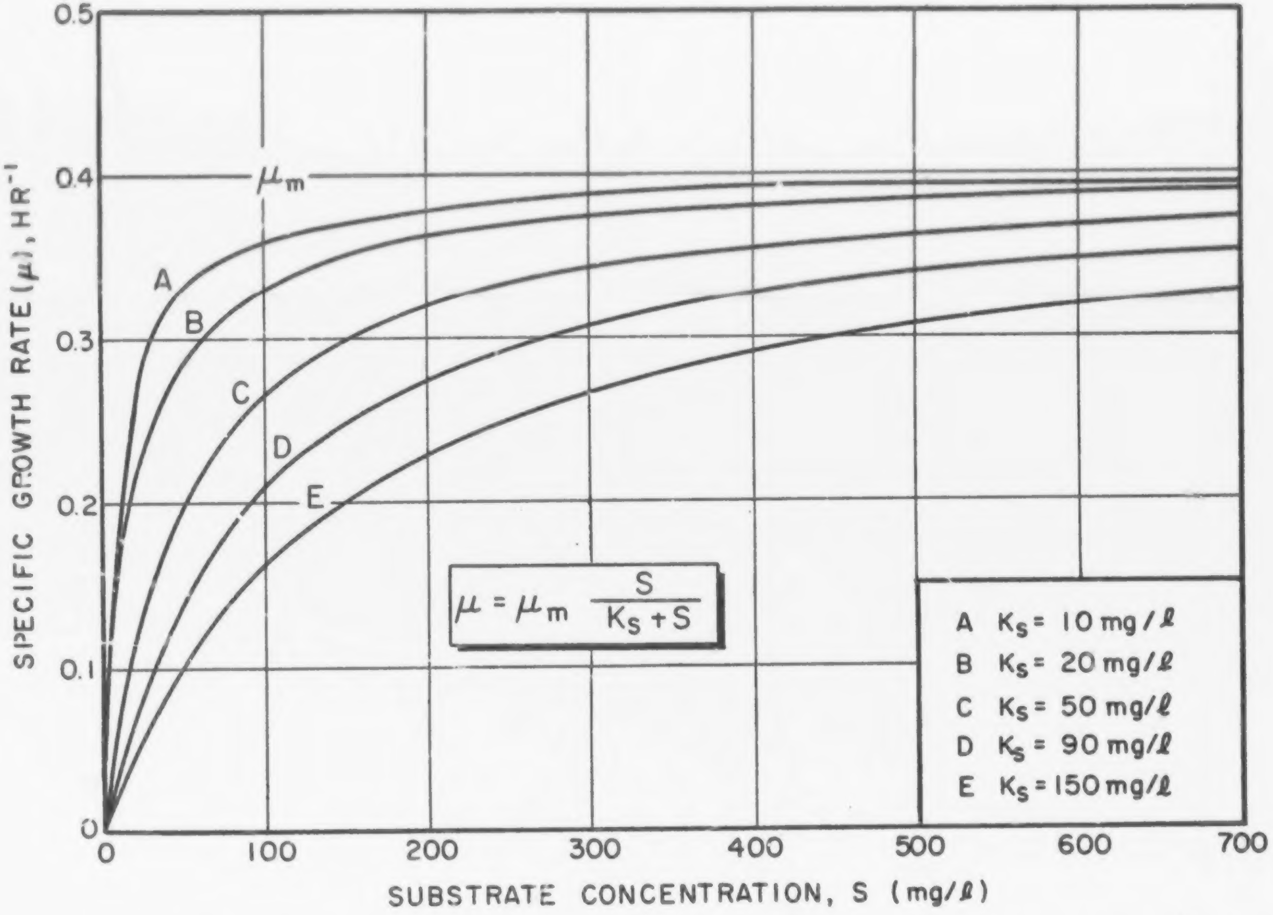

Fig. 10 PLOT OF THE MONOD EQUATION FOR μ_m OF 0.4 hr^{-1} AT VARIOUS VALUES OF K_S.

line up to a value of μ slightly in excess of 0.3 without significant differences in the corresponding values of s. In the latter case, both models might be expected to give comparable predictions of s, whereas in the case of curve E there would be a large difference in the predicted values of s, and a considerably larger difference in values of μ_m. Thus, if the system behaved in accordance with curve A, the selection of either the Monod type expression or the linear two-phase expression would appear to make little practical difference in defining the relationship between μ and s. However, for systems with larger k_s values, the numerical difference between the two predictions is increased. Concerning the use of either relation between μ and s, it is also important to note that in the lower ranges of s, i.e., s values of 25-75 mg/l, any of the curves predicted by the Monod expression could be plotted as a straight line without serious error. Since waste water treatment plants, to be effective, must yield effluent of at least this quality (preferably better), a linear relation could, for practical reasons, be assumed and, indeed, the two-phase concept was originally proposed on this basis. As previously stated, the general trend of the mass of the results presented in this study indicate that experimental data pertinent to the relation between μ and s support the use of the Monod equation. Therefore, even though at the lower substrate ranges and at low k_s values a linear relationship could be used, it cannot be recommended, since it does not fit the entire range of μ and s levels for any particular values of μ_m and k_s and only approximates a fit in special cases. It surely cannot be argued that the heterogeneous population is such a special case. Since the bulk of the data of this study fit the curvilinear expression, and since the same is true of data for most pure culture studies, it must be considered preferable to the linear function.

Furthermore, the curvilinear function which appears to be most applicable to heterogeneous populations, is the Monod relationship rather than the modifications of it which have been proposed; e.g., Teissier, Moser, etc. It is not intended to indicate that modification of the Monod equation may not be desirable, and indeed necessary, to make it more theoretically valid, but for the case of heterogeneous populations, one must expect variations in the growth parameters μ_m and k_s, and in cell yield as well. Refinement of the Monod equation does not seem warranted from a standpoint of practical application.

It should be emphasized that the utility of a kinetic model depends upon the experimental determination and insertion into the model of various "kinetic constants." In the case of the Monod model, the constants required are μ_m, k_s, and Y. It may be discerned from the present results that precise constancy is assuredly not characteristic of heterogeneous populations. Referring to Table II it can be seen that there was considerable variation in the values of k_s, μ_m, and Y at the various dilution rates which were studied. Taking as the best representation of average values, the averages of columns 12, 13, 14, and 15, the values of the growth parameters are μ_m, 0.53 hr^{-1}; k_s, 90 mg/l; Y_B, 0.45 mg/mg substrate; and Y_C, 0.65 mg/mg substrate. In general, μ_m values were lower for systems operated at the lower dilution rates. This result is reasonable, since operation at the higher dilution rates would be expected to select against slower-growing organisms. There was no discernible trend with dilution rate in the variations of k_s. The yield values obtained for the batch experiments were consistently lower than those obtained directly from the continuous flow reactor. Large variations in cell yield for heterogeneous populations grown on glucose have been reported by Rao and Gaudy (11), and the lack of constancy in yield was shown to be attributable, at least in part, to changes in predominant microbial species. The average values of Y observed in the previous study for yields obtained in batch agreed closely with the values for Y_C obtained in the present study. In the previous study, cells were not originally grown in continuous culture, but in batch reactors fed each 24 hours. There is no immediately apparent explanation for the difference in Y_B and Y_C obtained in the present study. It should be noted, however, that the average Y_C values for each dilution rate are based upon many more analyses than are the Y_B values. Also, differences in Y_B and Y_C have been observed by other investigators. Herbert, et al. (1) reported agreement between yields in batch and continuous flow at low dilution rates, but at higher dilution rates yields in continuous flow decreased. Cells for batch experiments were not taken from the continuous flow unit in their studies.

Concerning the attainment of steady state operation in completely mixed continuous flow reactors using heterogeneous populations, it was seen (Figures 6 and 7) that such a condition was approached with respect to COD and substrate, but not with respect to biological solids concentration in the reactor. However, it is important to note that even with the only approximate attainment of a "steady state," and with the variations observed in μ_m, k_s and Y, it was possible, using average values of μ_m and k_s obtained in batch and average values of Y_C obtained directly from the continuous flow reactor, to predict with a fair degree of accuracy the concentration of biological solids and substrate at dilution rates of practical significance in biological waste water treatment (see Figure 8). It is seen from Figure 8 that curves A, B, and C provide relatively good predictions of cell and substrate concentration in the reactor effluent up to dilution rates at which cell dilute-out becomes accelerated. It should be noted that disagreement between theory and observation at high dilution rates has been observed also in pure cultures and attributed to very small deviations from perfect mixing (1).

It can be discerned from Figure 8 that for effective substrate removal (for systems in which cells are not recycled) the operating dilution rate should lie below one-half of μ_m, i.e., a detention time of approximately four hours for heterogeneous populations using a readily-available carbohydrate waste under conditions where the carbon source is the limiting nutrient. In general, k_s values of 75-125 mg/l and μ_m values of 0.5-0.6 hr^{-1} can be expected with yield coefficients approximating 0.6. The present results would apply as a possible guide for those industrial wastes which consist primarily of carbohydrate with a non-limiting supply of ammonia nitrogen, e.g., pulp and paper and beet sugar wastes.

It is emphasized that the present study refers to the general applicability of the Monod model relating μ and s, and the attainability of steady state conditions in completely mixed reactors for heterogeneous populations in systems in which sludge recycle is not practiced. The average values for kinetic constants for systems employing cell recycle may be expected to differ from those herein reported, because of possible differences in the physiological state of the cells which are forced to exist at concentration levels higher than those which are determined by cell yield and the level of incoming substrate concentration. Indeed, the average values which are suggested for the kinetic constants in this paper are put forth with a considerable note of caution. It is seen that for heterogeneous populations, a considerable degree of variation in the numerical value of the kinetic constants was observed, and indeed, must be expected. The suggested values are based upon long term observation and repetitive analyses which are experimental criteria which must be observed when dealing with heterogeneous populations. Also, it is important to note that in the present study only minor and, hopefully, negligible changes occurred in the environment, i.e., temperature, pH, influent substrate concentration, etc., were maintained nearly constant. The effect of cell recycle and changes in the environment are presently the subject of a long term study in our laboratory.

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